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A Plan to Develop and Implement a Quality Assurance Program for the Ames/Salmonella Test

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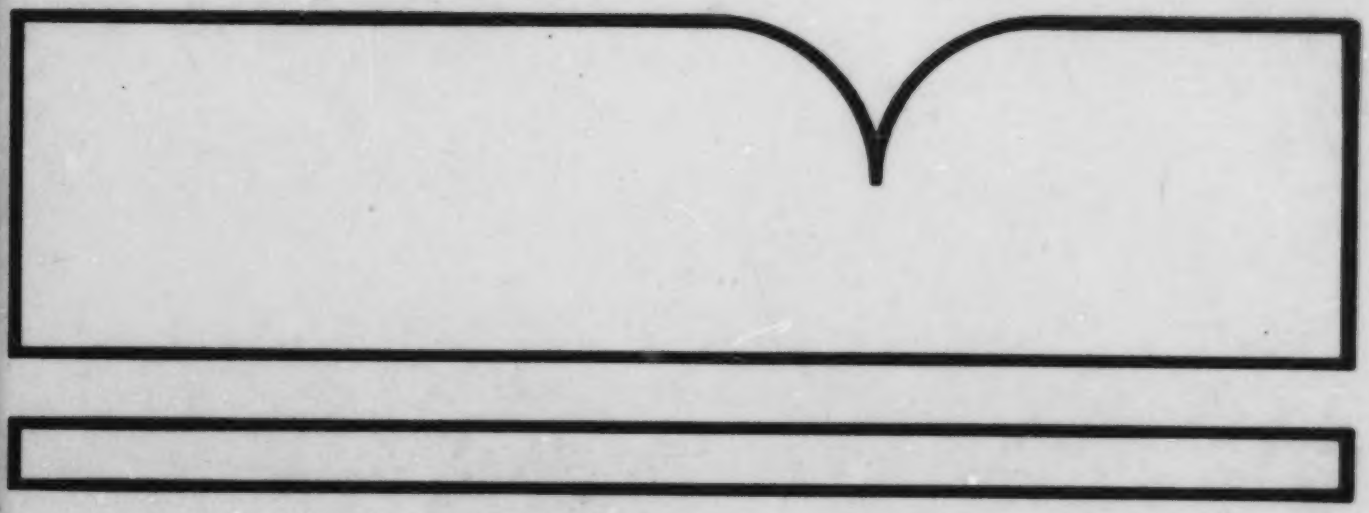

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A PLAN TO DEVELOP AND IMPLEMENT A QUALITY ASSURANCE PROGRAM FOR THE AMES/SALMONELLA TEST

by

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16. ABSTRACT

This document discusses a plan to develop and carry out a quality assurance program for the Ames/Salmonella test. The Ames test is in one of the 13 categories of tests mentioned in the Federal Insecticide, Fungicide, and Rodenticide Act legislation and is chosen as a model for presenting a QA program for all test categories.

There are three main sections contained herein. An introductory section describes the laws requiring this testing and the objectives of this quality assurance plan. The second section defines quality assurance terms, describes applicable quality assurance methods, and discusses the mailable audit materials approach that was chosen for these purposes. The third section details the mailable audit materials program and discusses selection of chemicals, preparation of samples, shipment of audit samples, pilot-scale audits, scheduling, other audit programs as models, data gathering procedures, data analysis, reporting of results, and costs.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The widespread use of pesticides has prompted the development of tests to aid in the registration, classification, and control of pesticides. This report discusses a quality assurance program for the Ames/Salmonella test. The Ames test is in one of the thirteen categories of tests mentioned in legislation regulating pesticides and was chosen as a model for presenting a QA program for all test categories.

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ABSTRACT

This document, prepared for the Office of Pesticide Programs, discusses a plan to develop and carry out a quality assurance program for the Ames/ Salmonella test. The Ames test is in one of the 13 categories of tests mentioned in the Federal Insecticide, Fungicide, and Rodenticide Act legislation and is chosen as a model for presenting a QA program for all test categories.

This document contains three main sections. An introductory section describes the laws requiring this testing and the objectives of this quality assurance plan. The second section defines quality assurance terms, describes applicable quality assurance methods, and discusses the mailable audit materials approach that was chosen for these purposes. The third section details the mailable audit materials program and discusses selection of chemicals, preparation of samples, shipment of audit samples, pilot-scale audits, scheduling, other audit programs as models, data gathering procedures, data analysis, reporting of results, and costs.

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SECTION 1

INTRODUCTION

This document is organized into three main sections. An introductory section describes the laws requiring this testing and the objectives of this quality assurance plan. The second section defines quality assurance terms, describes applicable quality assurance methods, and discusses the approach, a mailable audit materials approach, that was chosen for these purposes. The third section details the mailable audit materials program and considers the following aspects: selection of chemicals, preparation of samples, shipment of audit samples, pilot scale audits, scheduling, other audit programs as models, data gathering procedures, data analysis, reporting of results, and costs.

STATUTORY AUTHORITY

Under the Federal Environmental Pesticide Control Act of 1972,*[1] Section 3(c)(2)(A) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) provides that "the Administrator shall publish guidelines specifying the kinds of information which will be required to support registration of a pesticide product and shall revise such guidelines from time to time." The Act generally states in Section 3(c)(5) that the EPA Administrator shall not register a pesticide if he determines that the pesticide would cause unreasonable adverse effects on the environment. This phrase is defined to mean "any unreasonable risks to man or the environment taking into account the economic, social and environmental costs and benefits of the use of any pesticide."[2]

On July 3, 1975, the Administrator promulgated a regulation which provided general guidance for the registration, re-registration and classification of pesticides as required by the Act.[3] This promulgation, however,

---

*This Act amended and superseded the Federal Insecticide, Fungicide and Rodenticide Act, Pub. L.88-305, of 1972. The acronym FIFRA, however, is still in popular usage, so we will use it in this report.

did not include specific guidelines for toxicological evaluations of the pesticide products to be registered. On June 25, 1975, the Administrator separately proposed a rule-making which would, upon promulgation, provide for specific guidelines for the toxicological evaluations to be made to register, re-register, or classify a pesticide.[4] In response to the 1975 proposed guidelines for registering pesticides in the United States, the Administrator received over 80 comments from the interested public. In response to these comments, the Administrator greatly expanded the specificity of the guideline requirements in contrast to the 1975 proposal.[5] At present and until the August 22, 1978, guidelines are promulgated as final regulations, the requirements for registration and re-registration will be determined on a case-by-case basis. The EPA does, however, "consider these proposed guidelines as a general statement of policy regarding the nature and extent of data required to support a pesticide registration."[5] Therefore, a prudent registrant should pay careful attention to the protocols found therein.

Under the proposed guidelines, mutagenicity testing is required of a pesticide product under any of the following criteria:

> (i) The use for which registration application is made requires a tolerance or an exemption from the requirement to obtain a tolerance, or requires issuance of a food additive regulation; or
>
> (ii) The pesticide product is likely to result in a significant human exposure by dermal or inhalation routes before or during the normal reproductive portion of a human lifespan; or
>
> (iii) The active ingredients of any of its (their) metabolites is structurally related to a known mutagen or belongs to any chemical class of compounds containing known mutagens.[6]

Under the proposed guidelines, hazard evaluation for mutagenic potential and the active ingredients of a pesticide product shall be based upon a battery of eight different kinds of tests.[6]

In the preamble to the proposed rules, this requirement is described as follows:

> The proposed mutagenicity guidelines contain a battery of thirteen different kinds of tests which are grouped into three different kinds of categories. Two of the categories would be designed to evaluate the two large categories of genetic abnormalities: genetic mutations and

chromosome-aberrations. A third category of test would evaluate the potential of a chemical to interact with or cause primary change to DNA or other genetic processes in a cell. These tests detect a potentially broader spectrum of genetic events than do gene and chromosomal mutation tests.

An applicant would be required to submit data from several kinds of tests in each category: three kinds of gene mutation tests, and two kinds of damage/repair tests. Data would be required, therefore, from a total of eight different kinds of tests.[7]

Figure 1 illustrates schematically when testing is required, the testing requirements, and the analysis of results.

The mutagenicity test battery set forth in the proposed guidelines of August 22, 1978, includes a number of *in vitro* mutagenicity tests.[6] Among the *in vitro* tests referenced in the appendix to the guidelines is the Ames/*Salmonella* test for mutagenicity testing of chemicals.[8]

GENERAL OBJECTIVES

In compliance with the FIFRA legislation to assure safety to humans and the environment, companies applying to the Agency to register new products or to reregister existing products for pesticide use submit data on test results from the previously mentioned *in vitro* bioassays to the Office of Pesticide Programs (OPP). The objective of this quality assurance plan is to present a means of assuring that the laboratories submitting data to the OPP in support of claims for safety can produce information that is reliable and valid.

This quality assurance plan for the Office of Pesticide Programs (OPP) is a short-term interim goal to assist the OPP in evaluating the quality of data currently being received in its registration program, but there are several long-term goals complementary to and interfacing with this program. The Environmental Monitoring and Support Laboratory (EMSL) of EPA in Las Vegas is directing an Agency-wide cooperative effort to develop quality assurance programs for biological measurement activities within EPA or contractors to EPA. The Office of Research and Development (ORD) of the EPA is responsible for developing an Agency-wide quality assurance program and for coordinating its implementation. The Health Effects Research Laboratory of the EPA at Research Triangle Park, through the Genetic Toxicology (GENE-TOX) program,[9] is conducting detailed reviews of each of the *in vitro* test systems. One

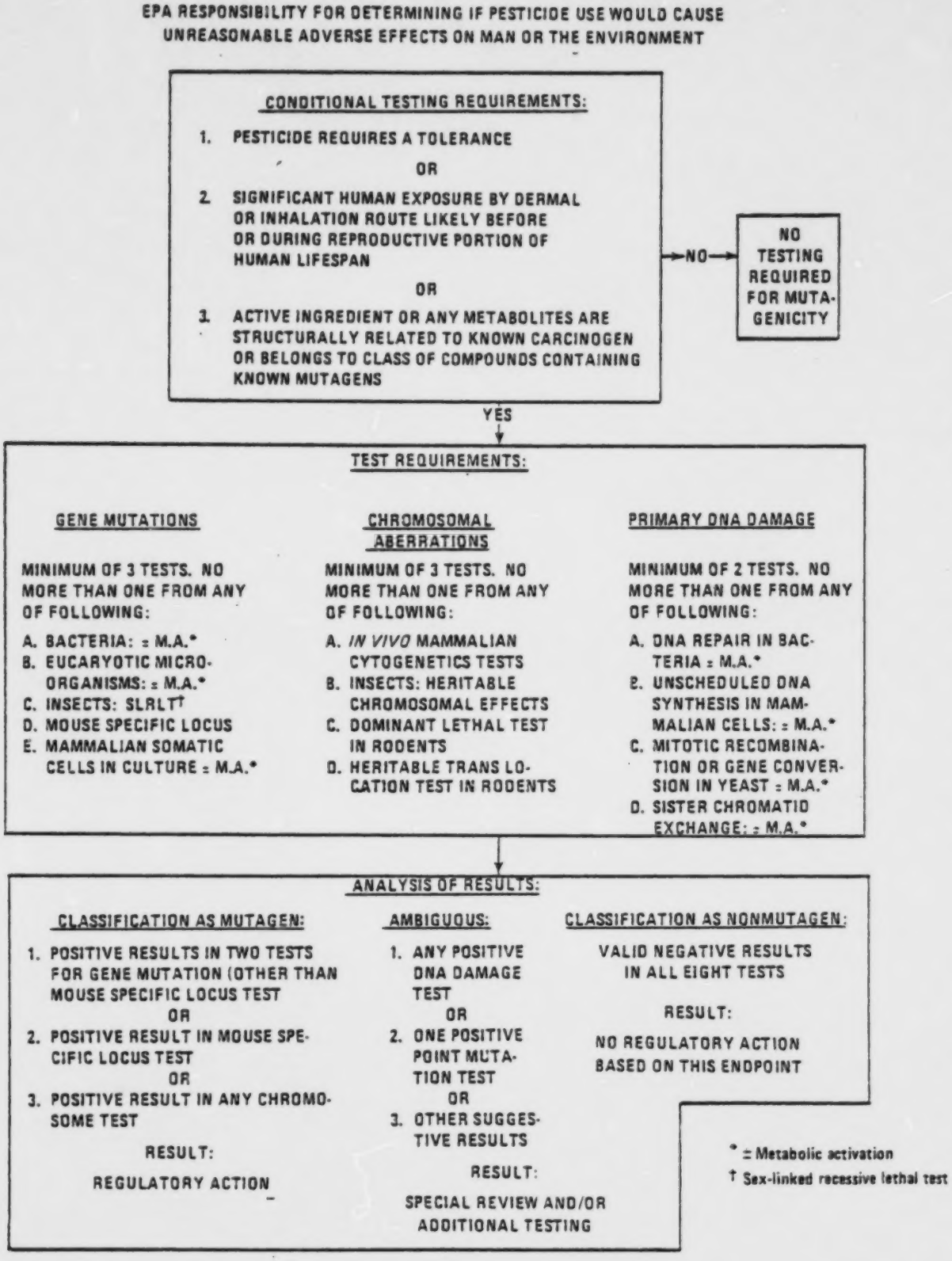

EPA RESPONSIBILITY FOR DETERMINING IF PESTICIDE USE WOULD CAUSE UNREASONABLE ADVERSE EFFECTS ON MAN OR THE ENVIRONMENT
CONDITIONAL TESTING REQUIREMENTS:
1. PESTICIDE REQUIRES A TOLERANCE
OR
2. SIGNIFICANT HUMAN EXPOSURE BY DERMAL OR INHALATION ROUTE LIKELY BEFORE OR DURING REPRODUCTIVE PORTION OF HUMAN LIFESPAN
OR
3. ACTIVE INGREDIENT OR ANY METABOLITES ARE STRUCTURALLY RELATED TO KNOWN CARCINOGEN OR BELONGS TO CLASS OF COMPOUNDS CONTAINING KNOWN MUTAGENS
NO
NO TESTING REQUIRED FOR MUTAGENICITY
YES
TEST REQUIREMENTS:
GENE MUTATIONS
MINIMUM OF 3 TESTS. NO MORE THAN ONE FROM ANY OF FOLLOWING:
A. BACTERIA: ± M.A.*
B. EUCARYOTIC MICROORGANISMS: ± M.A.*
C. INSECTS: SLRLT†
D. MOUSE SPECIFIC LOCUS
E. MAMMALIAN SOMATIC CELLS IN CULTURE ± M.A.*
CHROMOSOMAL ABERRATIONS
MINIMUM OF 3 TESTS. NO MORE THAN ONE FROM ANY OF FOLLOWING:
A. IN VIVO MAMMALIAN CYTOGENETICS TESTS
B. INSECTS: HERITABLE CHROMOSOMAL EFFECTS
C. DOMINANT LETHAL TEST IN RODENTS
D. HERITABLE TRANS LOCATION TEST IN RODENTS
PRIMARY DNA DAMAGE
MINIMUM OF 2 TESTS. NO MORE THAN ONE FROM ANY OF FOLLOWING:
A. DNA REPAIR IN BACTERIA ± M.A.*
B. UNSCHEDULED DNA SYNTHESIS IN MAMMALIAN CELLS: ± M.A.*
C. MITOTIC RECOMBINATION OR GENE CONVERSION IN YEAST ± M.A.*
D. SISTER CHROMATID EXCHANGE: ± M.A.*
ANALYSIS OF RESULTS:
CLASSIFICATION AS MUTAGEN:
1. POSITIVE RESULTS IN TWO TESTS FOR GENE MUTATION (OTHER THAN MOUSE SPECIFIC LOCUS TEST
OR
2. POSITIVE RESULT IN MOUSE SPECIFIC LOCUS TEST
OR
3. POSITIVE RESULT IN ANY CHROMOSOME TEST
RESULT:
REGULATORY ACTION
AMBIGUOUS:
1. ANY POSITIVE DNA DAMAGE TEST
OR
2. ONE POSITIVE POINT MUTATION TEST
OR
3. OTHER SUGGESTIVE RESULTS
RESULT:
SPECIAL REVIEW AND/OR ADDITIONAL TESTING
CLASSIFICATION AS NONMUTAGEN:
VALID NEGATIVE RESULTS IN ALL EIGHT TESTS
RESULT:
NO REGULATORY ACTION BASED ON THIS ENDPOINT
* ± Metabolic activation
† Sex-linked recessive lethal test

Figure 1. Proposed new mutagenicity testing requirements.

goal of the GENE-TOX program is the development of standard protocol reviews for these tests.

SPECIFIC OBJECTIVES

Of all the tests mentioned in the FIFRA legislation, the greatest amount of information is available on the Ames/Salmonella bacterial mutagenicity test. Although various modifications, such as the spot test,[10] the preincubation test,[11] and the well test,[12] are recognized and are applicable for special testing circumstances, the Ames test is used here to refer to the plate incorporation test[13] with the standard mutant tester strains, TA1535, TA1537, TA1538, TA98, and TA100, obtained from Dr. Bruce Ames at the University of California, Berkeley.

The Ames test is also the most widely used test of those mentioned in FIFRA.[14] Several large studies regarding use of the Ames test have been conducted.

Commoner and his associates at Washington University conducted a study on 100 chemicals including 50 presumptive carcinogens (materials which cause cancer in laboratory animals) and 50 noncarcinogens.[15] They found that the Ames test detected 41 of the carcinogens and correctly identified 49 of the noncarcinogens.

Dunkel and her associates at the National Cancer Institute analyzed 23 chemical compounds in a collaborative study of the Ames test in three laboratories.[16] Standard dosage levels and standard testing protocols were established so that results could be compared easily. In this study, 8 compounds were uniformly negative, 13 compounds were uniformly positive, and 2 compounds were positive in two laboratories and negative in one laboratory, for an overall agreement of 91 percent.

Grafe and Vollmar in Germany studied three chemicals in 19 laboratories.[17] Protocols and dosages were not standardized; they found a great variance for test results among laboratories.

Holstein and McCann at Berkeley studied 72 compounds in a comparison of test systems for detection of mutagenic effects.[18] They noted that "the Ames/Salmonella test is one of the few short-term tests in which a large number of carcinogens and noncarcinogens comprising a wide variety of chemical classes, have been tested . . . ." The Health Effects Research Labora-

tory (HERL) of the EPA at Research Triangle Park has also used the Ames test to assess the potential mutagenicity of a variety of pesticides.[19] [20]

These studies showed that the Ames test was a good screening test for carcinogens and mutagens but that quantitative results on the same sample varied greatly from laboratory to laboratory. Qualitative results were somewhat less disparate. The need for standard reference materials and for standard protocols was emphasized. For these reasons, the Ames test was selected as a prototype system for a quality assurance plan that could also encompass the other *in vitro* test systems.

SECTION 2

QUALITY ASSURANCE METHODS

DEFINITIONS

Several specific terms used to describe a program for the assurance of data quality for these bioassays are defined in the following subsections.

Quality

The total features and characteristics of a product or service that bear on its ability to satisfy a given need.[21]

Quality Assurance

Quality assurance is a system of activities whose purpose is to provide assurance that the overall quality control job is, in fact, effective. The system involves a continuing evaluation of the adequacy and effectiveness of the overall quality control program (see "quality control") with a view to having corrective measures initiated where necessary. For a specific product or service, this involves verifications, audits, and the evaluation of the quality factors that affect the specification, production, inspection, and use of the product or service.[21]

Quality Control

Quality control is the overall system of activities whose purpose is to provide a quality of product or service that meets the needs of users; also, the use of such a system.

The aim of quality control is to provide quality that is satisfactory, adequate, dependable, and economic. The overall system involves integrating the quality aspects of several related steps, including the proper specification of what is wanted; production to meet the full intent of the specification; inspection to determine if the resulting product or service is in accordance with the specification; and review of usage to provide for revision of specification.

The term "quality control" is often applied to specific phases in the overall system of activities, as, for example "process quality control." Broadly, quality control has to do with making quality what it should be, and quality assurance has to do with making sure that quality control is what it should be. In some industries, quality assurance is used as an all-inclusive term combining both functions.[21]

Accuracy

The relationship of individual measurements to a known and verified reference value.[21]

Precision

Degree of mutual agreement (or reproducibility) among individual measurements. Relative to a method of test, precision is the degree of mutual agreement among individual measurements made under prescribed like conditions.[21]

APPLICABLE QUALITY ASSURANCE METHODS

Several QA methods are applicable to a quality assurance program for the Ames test. Approaches that might be considered here are the use of split-sample schemes, the use of onsite systems audits, and the use of validated, mailable audit samples. At the present time, data are considered valid if the laboratory report is signed by an authorized representative of the testing group. This "signature audit" is considered to be the only approach available to the Agency given the state of the science to date.

In a split-sample scheme, audit (unknown) materials are divided among a number of laboratories (without any preliminary analysis for validation). Results are collected, and the mean and variance computed. Laboratories are ranked or evaluated according to the difference between the laboratory's value and the mean of the reported values from all laboratories. Precision (reproducibility) but not accuracy can be evaluated.

Systems (or qualitative) audits consist of evaluations, principally through inspection, of the various components of an operation. Factors evaluated might include organization of the laboratory, planning, training, sample collection, maintenance, calibration procedures, analytical methods, reporting procedures, and quality of chemicals and other materials used in a

test. Checklists are often used in systems audits to compare suitable laboratory practices with those actually being carried out. Scoring schemes may be devised to quantify results of a qualitative audit.

Quantitative audit sample schemes involve the preparation of a set of materials whose composition and properties in the test system are known to the auditor. These materials may be tested by the auditor prior to their use. A statistically valid test plan is devised. Samples can be mailed to laboratories as identified audit materials, with instructions for reporting results, or these audit samples can be included as coded blind samples with a group of samples submitted for routine testing (concomitant audit sampling). The latter method is superior as an evaluative tool in that it assesses a laboratory's routine, daily performance; the former method probably represents a laboratory's best efforts. A laboratory's reported results are compared with expected results and with the results reported by other laboratories participating in the audit.

SELECTION OF METHODS

The use of mailable materials clearly identified to the laboratory being evaluated as audit materials has been selected as the best approach immediately implementable because:

1. Many laboratories are submitting data; therefore, onsite evaluations would be costly and very time consuming.

2. Neither systems audits nor split-sample schemes would provide an evaluation of a laboratory's accuracy. In the case of split-schemes, in the worst instance where there were large variances among laboratories, no information useful for this task might be obtained. Further, there is no practical way to insert blind samples into a laboratory's analysis stream.

3. There is no widely accepted standard method for all aspects of performing the Ames/Salmonella test. Parts of the systems audit are geared toward comparing an individual laboratory's performance to well-established standards and protocols. This lack of a validated protocol would also preclude a formal certification program, although results of previously mentioned collaborative and evaluative studies certainly indicate the need for a standardized protocol.

The details of the mailable audit scheme for the Ames/Salmonella test will be discussed in the next section.

SECTION 3

THE MAILABLE AUDIT MATERIALS PROGRAM

A variety of factors will be considered in the mailable audits program: (1) selection of chemicals, (2) preparation of samples, (3) shipment of samples, (4) pilot-scale audits, (5) data gathering procedures, (6) analysis of data, (7) schedule of audits, and (8) reporting of results. These topics are discussed in subsequent sections.

SELECTION OF CHEMICALS

There are several factors to consider in selecting audit materials for a mailable program for the Ames/Salmonella test. There should be some samples that give a positive (mutagenic) response and some materials that are clearly negative (nonmutagenic). Some of the materials should be promutagens, which require metabolic activation before a positive response is shown. The physical characteristics of the chemicals should be known; for the purposes of this audit, materials with low vapor pressure are preferable. Solubility should be known so that problems of precipitation can be avoided. The materials should not be extremely heat- or light-sensitive, nor should materials be extremely reactive under possible shipment and testing conditions. For example, sodium azide, a mutagen that presents an explosion hazard,[22] would not be a suitable choice.

Some pure compounds and some mixtures of compounds would be suitable audit materials. A set of samples with varying levels of complexity would best evaluate the capabilities of a testing laboratory. Such a set of audit samples might include:

1. A direct-acting compound in a solvent--This would be the simplest material to analyze and would assess the minimum level of competence in performing the test. Candidate compounds could include the usually employed positive control materials for the Ames test, such as methyl methanesulfonate, 9-aminoacridine, 4-nitro-o-phenylenediamine, 2-nitrofluorene, hycanthone methanesulfonate, or nitrofurantoin.[23]

2. An indirect-acting compound (promutagen) in a solvent--At the next level of complexity, this type of sample would challenge the metabolic (S-9) activation mixture as well as the rest of the test system. Again, the appropriate set of positive controls would be candidate compounds; these include 2-aminoanthracene or dimethylbenzanthracene.[23]

3. A mixture of pure, direct-acting compounds in the appropriate solvent(s) for activating only frameshift or only base-pair sensitive strains--Such a sample would check strain specificity. By the addition of chemically similar, nonmutagenic materials or dyes, the mixture could be formulated so that chemical analysis would be difficult.

4. A mixture of pure, direct-acting compounds that elicit a positive response in all five tester strains--Such a mixture could be formulated as in 3. so that chemical analysis is difficult.

5. A mixture of pure compounds that require metabolic activation--This category of sample might include compounds that are deactivated by higher levels of S-9 but show a strong response at lower levels. Benzo-a-pyrene shows such characteristics.[14]

6. A complex mixture/environmental sample--Coal tar, various sources of which have been extensively tested, is a possible choice in this category.[24]

7. A mixture that requires chemical fractionation to show mutagenicity--This type of sample would be the most difficult to analyze. As a whole sample, in the Ames/Salmonella system, there would be no apparent mutagenic activity. However, chemical fractionation would separate out highly mutagenic subfractions. Certain fly ash samples and air particulate samples meet the specifications of this category.[12]

8. Nonmutagenic materials--An important characteristic to be assessed in an audit would be the ability of a testing laboratory to obtain correct negative results. The negative, nonmutagenic samples could be single compounds or mixtures. To avoid the possibility of easy chemical analysis, dyes or nonmutagens that are structurally similar to mutagens could be added in the formulation of these samples.

Even though compounds for audit samples could be chosen from available references, EPA laboratory verification of the set of audit materials would be necessary before initiation of the audit program for several reasons. First, such an in-house verification would be a first-line check against mistakes in formulation. Such a check would also ensure that the audit materials did not contain trace components that might alter the biological

response, as in the recent case with Agent Orange.[25] A preaudit verification would be especially important for sample classes involving novel mixtures of compounds, because the preaudit test would detect any synergistic or antagonistic relationships among the components.

The chemicals chosen as reference standards in this audit program will probably be obtained from commercial sources. It is recognized that the usual source of primary standards for chemical analysis, such as the National Bureau of Standards[26 27] and EPA's Environmental Monitoring and Support Laboratory[28] do not, at present, stock standards that are appropriate for this audit.

PREPARATION OF SAMPLES

Some important quality assurance considerations in the actual formulation of audit materials are homogeneity of mixtures, choice of solvents, physical state (solid or liquid), choice of storage conditions prior to shipment, and stability.

When batches of a substantial size are prepared, the homogeneity of a mixture can be determined by the ASTM cone and quarter method.[29] In this method, the batch is sequentially subdivided and a fixed pattern of the resultant portions is chosen for analysis. Analytical results should not vary more than replicates on the same sample.

The choice of solvents will depend directly on the choice of chemical compounds. Consideration should be given to solubilities of pure compounds, compatibility with the agar-aqueous test system, and the avoidance of toxicity to the bacteria and S-9 metabolic activation system. Although many solvents may be used for initial dissolution of the sample, with subsequent solvent exchange, dimethylsulfoxide, ethanol, and water are preferred for dosing into plates. Chemicals will be shipped in the most stable form.

Laboratories that prepare and test the audit materials should follow the guidelines described in Guides for Quality Assurance in Environmental Health Research, Health Effects Research Laboratory, Research Triangle Park, NC.[30] Figure 2, taken from this document, describes the general classifications of information that should be compiled by the testing laboratory prior to initiation of the task. Subsequent experimental records should

(A) A descriptive title and statement of the purpose of the study.

(B) Identification of the test and control substance by name, chemical abstract (CAS) number, or code number.

(C) The name and address of the sponsor including the sponsor's project manager. The name and address of the testing facility at which the study is being conducted.

(D) The proposed starting and completion dates.

(E) Justification for selection of the test system.

(F) Where applicable, the number, body weight, range, sex, source of supply, species, strain, substrain, and age of the test system.

(G) The procedure for identification of the test system.

(H) A description of the study design, including the methods for control of bias.

(I) A description and/or identification of the diet used in the study as well as solvents, emulsifiers and/or other materials used to solubilize or suspend the test or control substances before mixing with the carrier. The description must include specification for acceptable levels of contaminants that are reasonably expected to be present in the dietary materials and are known to be capable of interfering with the purpose or conduct of the study if present at levels greater than that established by the specifications.

(J) The route of administration and the reason for its choice.

(K) Each dosage level, expressed in milligrams per kilogram of body weight or other appropriate units, of the test or control substance to be administered and the method and frequency of administration.

(L) Method by which the degree of absorption of the test and control substances by the test system will be determined if necessary to achieve the objectives of the study.

(M) The type and frequency of tests, analyses, and measurements to be made.

(N) The records to be maintained.

(O) The date of approval of the protocol by the sponsor and the signature of the study director.

(P) A statement of the proposed statistical methods to be used.

Figure 2. Proposed protocol contents, EPA.[30]

include "a written record (in ink, in a bound, page-numbered notebook) of equipment serial numbers, reagents and supplies used, animal identification and test data, as well as a record of equipment modification . . ."[30] Reference 31 lists the proposed rules for nonclinical laboratory records and reports.

SHIPMENT OF AUDIT SAMPLES

Because audit materials will be mailed, it is appropriate to consider suitable means of transportation. Title 49, Code of Federal Regulations (CFR),[32] covers all modes of transport of materials within the United States. Parts 100 through 177 specifically deal with the transport of hazardous materials. All materials considered hazardous are discussed concerning which means of shipment are allowed, what quantities can be shipped, and what packaging is required. Classes of hazardous materials discussed in Title 49 CFR include nonflammable and flammable gases, liquids, and solids; explosives; poisons; organic peroxides; corrosive materials; irritants; oxygen; pyrophoric liquids; oxidizers; and radioactive materials.

Three methods of transportation were considered for audit materials--Federal Express, United Parcel Service, and U.S. Mail. All three means of transport are governed by Title 49 CFR, but they vary in the additional regulations they impose on shippers and the services they provide to shippers.

Federal Express's Service Guide,[33] United Parcel Service's Guide for Handling Hazardous Materials,[34] and the United States Mail's Domestic Mail Manual[35] describe the services available from each organization. Details on shipping regulations are included in Appendix A.

Audit materials could be mailed as homogeneous mixtures of solids, with appropriate directions for dissolution and dosing, or as already prepared solutions either in concentrated or dilute form. The choice of physical state will depend on the stability of the chosen material in that physical state. Generally, solids will be more stable than concentrated solutions, and concentrated solutions will be more stable than dilute solutions. There will undoubtedly be exceptions to this general rule, and chemical and bioassay tests for the stability of the audit materials should be performed periodically. As was stated earlier, there will be attempts to choose audit

materials that are not rapidly degraded (e.g., during shipment) by heat, light, or moisture. Audit materials should be refrigerated, but testing should also be performed to ensure that no significant degradation occurs during one week at room temperature (20°-25° C).

PILOT SCALE AUDITS

When the sets of audit materials have been prepared, properly tested for mutagenic activity, and packaged for storage and shipment, a small-scale trial audit will be initiated. In this phase, the same audit sample sets and instruction packages will be sent to seven laboratories, to be selected by EPA. The trial audit will test many factors of the QA program: clarity of instructions, stability of materials during transit, time required for transit of samples, applicability of data entry forms, and schemes for data interpretation. With the selection of reputable and experienced laboratories, such a trial audit scheme would also furnish test results to further validate and collaborate the initial testing laboratory's results. This approach will also furnish valuable information on expected variances among results from a larger scale audit, such as that anticipated to be needed by OPP for all laboratories submitting Ames test data. Seven has been selected as the target number of laboratories for the small-scale audit because agreement among eight laboratories (seven plus the original testing laboratory) would constitute a statistically significant probability (at the 1 percent level) that the concensus answers obtained were, in fact, correct and not purely chance occurrences.

SCHEDULING OF AUDITS

When a laboratory makes an initial submission of bioassay data, audit materials will be mailed to evaluate that laboratory's capabilities. Because there are, at present, a large number of laboratories that have submitted data and because it is anticipated that laboratories may wish to participate in an audit in anticipation of submission of data to OPP, a regularly scheduled mailable audit materials program is recommended, with special mailings for new participants.

The mailable audits program described herein for the Ames/Salmonella test would, after an initial satisfactory performance on a set of audit

samples, provide a new set of audit samples each year. For laboratories that did not show acceptable performance, a second and then a third unique set of samples would be provided. If, after three attempts, a laboratory did not demonstrate satisfactory performance, that laboratory would have to wait one year before receiving another set of samples. This scheme would allow for the occasional problems encountered even in competent and good laboratories, while not burdening the organization preparing audit materials with the task of preparing and validating more than three sets of audit samples per year.

DATA GATHERING PROCEDURES

Several data systems that could be used at least indirectly for Ames/Salmonella test results have been or are being developed. Presently under development are the Health and Environmental Effects Data Analysis (HEEDA) system, the Toxicology Data Management System (TDMS), and the Chemical Substance Information Network (CSIN). The HEEDA system is being developed at the University of Pennsylvania for EPA/Washington and is being designed to handle data from a variety of test systems. The TDMS is being developed by the Food and Drug Administration at the National Center for Toxicological Research in conjunction with the National Cancer Institute of the National Institutes of Health; the TDMS will process data from a variety of bioassays and will include data analysis and reporting capabilities. This system did not originally contain provisions for Ames/Salmonella data but such provisions are being drawn up. The CSIN is being implemented to allow access to the other information systems and will direct users to sources of specific information within other data systems.

Already implemented data systems that can process information from the Ames/Salmonella test include PROPHET, the Environmental Mutagen Information Center (EMIC) data system, and the HERL-RTP data system. PROPHET is a data base management package for pharmacological and cancer research, developed by Bolt, Baranek, and Newman of Massachusetts for the National Institutes of Health. This system contains graphical and statistical capabilities. With a special programming addition, PROPHET is being used by NIEHS-RTP for Ames/Salmonella data. The EMIC, operated by Oak Ridge National Laboratory in Tennessee for the Department of Energy, is principally a literature

search and reference source; the EMIC does not record or analyze raw data. The HERL-RTP data system collects detailed, raw data from a variety of *in vitro* bioassay systems; this system provides statistical analysis and graphics capabilities.

Initially, the data entry system proposed for use is the system designed and in current use at HERL-RTP. Appendix B describes the data entry forms for this system and gives detailed instructions for entering data. Basically, the HERL data entry forms collect information on identity of the testing laboratory, sample identity, activation material, test type (adaptable to tests other than the Ames/*Salmonella* plate test), microorganism strain, any germane remarks, phenocopy checks, sterility checks on S-9, amount of activation mixture per plate, preincubation time and temperature, technician performing the tests, plate counts on positive and negative controls, dosages and counts on each plate, type of positive controls, toxicity data, and type of solvent.

Programs already implemented for the HERL data system will give a printout of all raw data, a printout of data for a given sample combining the experiments with and without metabolic activation, computations of mean and standard deviation, and a plotted dose/response curve for each experiment.

For the special purposes of this audit, a form will be added to the HERL data entry forms to collect the testing laboratory's evaluation (+/-) on the mutagenicity of each audit sample and the testing laboratory's ranking within each strain, with and without metabolic activation (from most mutagenic to least mutagenic) of the samples constituting an audit set.

HERL is designing data entry forms for collecting information on the metabolic activation procedures used in a test, and these forms, plus instructions for their use, will be added as an appendix when this information becomes available.

Although the protocols for performing the testing of audit samples will not be specified in this Quality Assurance Plan, certain information on how the testing was done will be useful in evaluating the data. Such protocol information may be helpful in improving future performance of individual laboratories and possibly in improving the general level of information about Ames testing. The questionnaire that will be used to gather information on

testing protocol is included as Appendix C. This questionnaire will be used on a one-time basis to gather information on a laboratory's methods of testing; this information will not be required with each set of test results.

DATA ANALYSIS

The HERL/RTP Pesticide QA Program, a Model Program

The interlaboratory control program for pesticide analysis conducted by the Quality Assurance section of the Chemistry Branch,[37] EPA-ETD/HERL Laboratory in Research Triangle Park, NC, serves as a model for this program. This program provides samples of unknown composition to a number of participating laboratories. Data submitted on standard forms by these laboratories are analyzed to assess the individual laboratory's capabilities in chemical analysis and also the laboratory's performance relative to all participants.

The initial step in data analysis is the completion of a summary table of results from all laboratories. After the outliers are eliminated from the data set, the mean, standard deviation, relative standard deviation, and total percent error for each compound are computed. Based on past experience, the group of results for each compound were evaluated as "good", "fair", or "poor", from relative standard deviation. An acceptable average total error value of 50 percent was adopted for this program.

The ranking scheme incorporates three criteria: the correct identification of compounds, the correct quantitation of compounds, and the reporting of compounds not present. A perfect score is 200 points--100 points for identification and 100 points for quantitation. Correct identification of a compound adds 100/n points, where n equals the number of compounds in the unknown sample; identification of a compound not actually present subtracts 100/n points. The point score for quantitation of an individual compound equals 100/n minus the absolute error divided by the standard deviation, as calculated on the summary sheeet, for that compound:

$$\begin{matrix}\text{COMPOUND} \\ \text{QUANTITATIVE} \\ \text{SCORE}\end{matrix} = \frac{100}{n} - \frac{\text{ABSOLUTE ERROR}}{\text{STANDARD DEVIATION}} .$$

The total quantitation score is the sum of the individual scores. Thus, if all laboratories do poorly, the quantitation score is equitably adjusted for individual laboratories.

Proposed System for Evaluation

Five major points will be used to evaluate performance in this quality assurance plan:

1. The mutagenic/nonmutagenic (+/-) determination by the testing laboratory as compared with the expected results. The expected results would be based on published response values for the chemicals chosen as audit materials and on the preliminary and pilot audit analyses.

2. The mutagenic/nonmutagenic (+/-) determination by the testing laboratory as compared with the +/- determination by a reviewer of the raw data. Mathematical models have been developed that consider all raw data (plate counts on controls and samples), factor in toxicity, and give an assessment of the mutagenic or nonmutagenic nature of the sample. These models are described in Appendix D.

3. The mutagenic/nonmutagenic (+/-) determination by the testing laboratory as compared to the +/- determination by other laboratories submitting data.

4. Positive and negative control values as reported by a testing laboratory and compared with the appropriate normal ranges. Normal ranges for controls will be determined from values published in a consensus report by NIEHS, from values determined in the preliminary tests and pilot audits program, and from a statistical analysis of the values reported by all laboratories submitting data.

5. Ranking by the testing laboratory of the set of audit samples, from the most mutagenic to the least mutagenic within each strain/S-9 combination of bacteria, with and without metabolic activation, compared to a similar ranking according to expected results. Again, expected results will be based on determination from preliminary and pilot studies and values reported by all laboratories submitting data.

It is proposed that the following weighting scheme be applied for evaluation of a laboratory's capabilities in the Ames/Salmonella test:

Criteria	Points
1	50
2	25
3	5
4	10
5	10
	100

This scheme gives the most importance to the laboratory's qualitative evaluation of test results, as was suggested in earlier referenced studies.[16 23] Depending on the quality of data reported by the various laboratories, it is acknowledged that this weighting scheme may require modification after the initial data are collected and analyzed.

Proposed System for Data Interpretation

The data interpretation model mentioned in Point 1 is an extension[38] of the Poisson model of Stead, Hasselblad, Creason, and Claxton[39]:

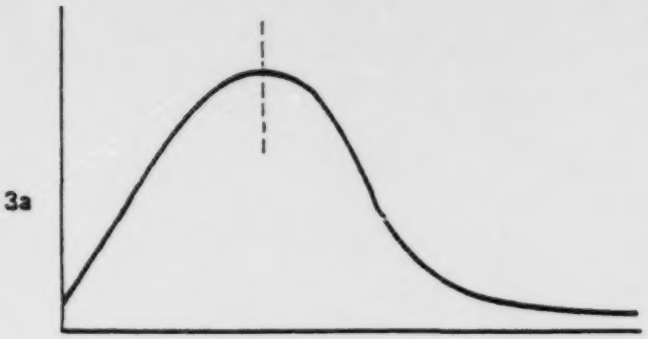
$$P(k \text{ revertants dose } d) = \exp(-\lambda(d))\ \lambda(d)^{k}/k! \ ,$$

$$\lambda(d) = (b_0 + b_1\ d^{b2})\ \exp(-\mu d) \ ,$$

for the number k of revertants observed at dose d. The mean number $\lambda(d)$ of revertants at dose d is modeled as a power function of dose attenuated by an exponentially decreasing function of dose to account for toxicity.

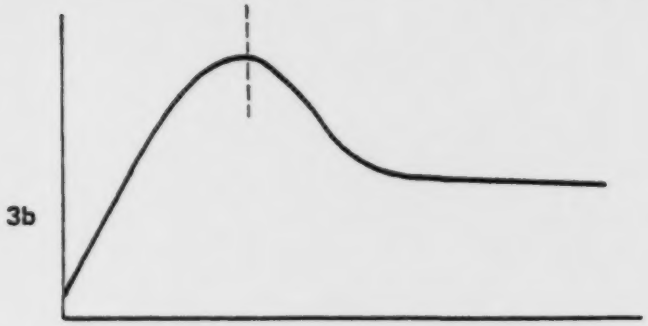
The previously mentioned extension of the model uses a package of Statistical Analysis System (SAS)[40] computer programs for the statistical analysis of Salmonella plate assay data. Each of the programs is based on a negative binomial probability distribution for the number of revertant colonies. The data analyst has the option of employing one of several parametrizations of the mean number m(x) of revertants at dose x. The choice of parametrization for each set of experimental conditions (compound, S-9, etc.) can be based on inspection of the plot of revertant counts versus dose. For data sets suggestive of mutagenicity, one of the shapes depicted in Figure 3 is generally appropriate. The associated parametrization of the mean for each case is included in Figure 3. Scientific rationale for these functional forms, as well as for the negative binomial distribution, is given in the manuscript of Appendix D. Occasionally the data exhibit a quadratic or possibly higher order effect at low doses. In such a case, x may be replaced by a power function of x, with exponent greater than one, after the fashion of Stead et al.[39] In each case, the mutagenicity decision is based on whether or not the initial slope S is significantly greater than zero at a given significance level (e.g., 5 percent or 1 percent).

The output from the statistical analysis procedures includes estimates of parameters, standard errors, covariances, 95 percent confidence intervals



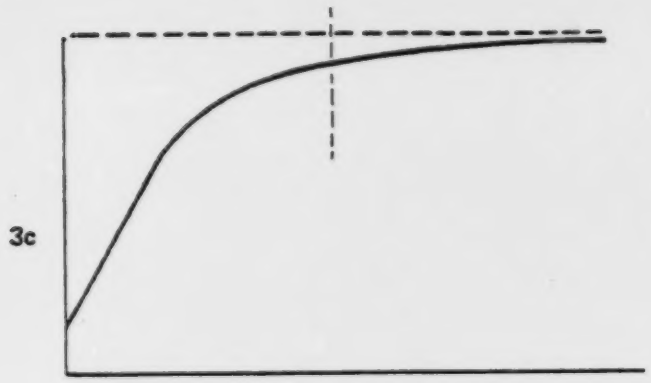
3a

$$m_2(x) = (B + Sx)e^{-Tx}$$



3b

$$m_2(x) = [B + V(1 - e^{-Sx/V})]e^{-T(1 - e^{-Sx/V})}$$



3c

$$m_3(x) = [B + V(1 - e^{-Sx/V})]$$

Figure 3. Some special cases of the general form

$$m(x) = E(N)\ P(s|x)\ P(r|s;x)$$

where

- $E(N)$ = the mean number of cells surviving the initial plating, available to reverse mutate or die of toxicity of the compound,
- $P(s|x)$ = the conditional probability of surviving the toxic effects of a dose x of the compound, given survival of the initial plating, and
- $P(r|s;x)$ = the conditional probability of a reverse mutation at dose x, given survival of the initial plating and the toxicity of the compound,
- B = the background mean numbers of revertants,
- S = the initial slope, and
- T = a toxicity rate constant.

for parameters, and plots of observed versus expected results. Some examples are presented in Appendix D.

In the context of this model, tests of toxicity reduce to tests on the parameter μ, while the compound is regarded as mutagenic if β_1 is significantly greater than zero. If toxicity is not evident or only weakly apparent, it may be best to set $\mu = 0$.

Tests that the laboratories are in reasonable agreement can be based on preliminary tests of the hypothesis that the respective parameters estimated from their data are not significantly different. If some of the parameters are not significantly different, then increased power can be obtained by pooling the data across laboratories, taking as constant the parameters that do not vary significantly across laboratories, and allowing other parameters to be specific to individual laboratories. For example, it would not be surprising to find spontaneous reversion rates for a given strain that vary across laboratories, while the toxicity effects and initial slope appear constant over laboratories. With a three-parameter model and L laboratories, 2 + L degrees of freedom (1 for slope, 1 for toxicity, L for background (intercept)) would be used in parameter estimation rather than the 3L degrees of freedom expended in treating each laboratory separately. Of course, sample size is also increased by pooling data from multiple laboratories. In this way, a more powerful test of mutagenicity can be obtained. If the data of one or two laboratories deviate markedly from the rest, these data will be omitted from the analysis.

REPORTING OF RESULTS

Five reports describing the results of the QA program are required.

First, a report of a laboratory's raw data, as encoded for interpretation, will be mailed to that laboratory. An executive summary of each testing laboratory's performance, based on the five evaluative criteria and the weighting scheme, will be prepared for the Office of Pesticide Programs. The report for each laboratory may also contain a technical discussion of problems if this is deemed appropriate by the reviewers. An annual report on the QA program will also be prepared for the OPP and HERL detailing all aspects of the program for each year. The annual report will contain summary statistics on the performance of all laboratories submitting data during a

yearly reporting period and will detail any problems (and the solutions) encountered in implementation of the QA plan.

A fourth report will be issued to each laboratory submitting data. This report will contain a copy of the data submitted by the laboratory for evaluation as well as notation of any modification made for the purposes of keypunching or programming. After the QA program is established and an appropriate amount of data has been collected, the reports to submitting laboratories will also contain a coded printout of the results of all submitting laboratories, with code identification for the particular laboratory. The individual laboratory report will also contain statistical analysis of the results of all submitting laboratories and comparisons with expected values, as in the previously referenced HERL pesticide analysis program.[37]

It is anticipated that these interlaboratory studies will be of interest to the general scientific community. A fifth category of reports will be the publication of results from the QA program in the appropriate scientific journals, e.g., Mutation Research and Journal of the American Statistical Association.

OUTLINE AND COSTS

Figure 4 shows an outline of the implementation scheme for the QA plan with estimated manhour requirements for the various steps. These costs will depend on a large number of factors, such as the competence of laboratories being audited and the volume of data reported; therefore, the costs should be considered rough estimates at best. For the purposes of costing, a 200-sample batch was estimated for the first set, 50 samples for the second set, and 20 for the third set. Considering all 270 samples and totaling costs, audit samples would cost \$520/6 = \$87 each. This compares with \$6.82 per sample (annual program cost of \$150,000 for 22,000 samples) for the chemical analytical samples furnished for pesticide analysis by HERL. However, the HERL sample cost includes only recordkeeping, storage, packaging, and mailing of commercially obtained bulk materials. At present, the HERL pesticide program does not have the facilities to test and verify composition and concentration of each lot of bulk pesticide. Audit samples for bioassay are not commercially available so an audit program for bioassays must also include cost of preparing and testing materials.

	Estimated Manhours
I. Preliminary Planning	
A. Selection of appropriate QA methods B. Submission and approval of a QA plan	This document, model development, and associated work ..... ($27,000)
C. Selection of chemicals	(by HERL/RTP)
II. Pilot Program	
A. Preparation of samples	500 hr
1. 200 sets of six samples for first round	
2. 50 sets of six samples for second round	
3. 20 sets of six samples for third round	
B. Testing of samples	1,000 hr
1. Initial tests	
2. Replicate tests	
3. Testing for storage stability	
C. Small-scale audit	
1. Packaging and mailing of samples to seven labs	160 hr
2. Compiling and evaluating data (+$5,000 computer time)	320 hr
3. Reporting of results	100 hr
4. Modifying protocol, if necessary	100 hr
III. Full-Scale Audit Program	
A. Packaging and mailing samples	300 hr
B. Compiling and evaluating data (+$10,000 computer time)	400 hr
C. Reporting of results	200 hr
1. To OPP	
2. To participating labs	
3. To scientific community	
D. Remailing of second (then, if required, a third) set of samples to labs that do not show acceptable performance	200 hr
	3,280 hr

Approximate cost/sample set = 3,280 hr × $30/hr = $98,400
Computer time = 15,000
I,A & I,B = 27,000

$$\frac{\$140,400}{270} = \$520/\text{sample set}$$

Figure 4. Outline of the audit program for Ames/Salmonella testing.

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APPENDIX A
SUPPLEMENTARY SHIPPING INFORMATION

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SUPPLEMENTARY SHIPPING INFORMATION

According to the Federal Express Service Guide, Federal Express limits the acceptance of and quantity of a hazardous material exactly in accordance with Title 49, CFR. The "Priority One" overnight air service is designed to provide special care and handling of certain materials defined as "hazardous for transportation purposes" by the Department of Transportation. The "Priority One" service will not accept packages exceeding 70 pounds or 108 inches in length and girth combined; 54 inches is the maximum acceptable length and 300 pounds is the maximum daily total shipment. These limitations would probably not be exceeded by small-quantity shipments of audit materials. The "Priority One" service will accept poison-labeled packages only on Mondays, Wednesdays, and Fridays. Carcinogens, etiologic (disease-causing) agents, and certain radioactive materials will not be accepted without advance arrangements by the shipper with the local Federal Express offices. A toll-free telephone information service at the Federal Express headquarters in Memphis, Tennessee, is provided (800-238-5355, ext. 3853). Pages 18 and 19 of the previously mentioned Federal Express Service Guide give detailed shipping information and are included in this appendix.

The United Parcel Service (UPS) published a detailed Guide for Handling Hazardous Materials, which summarizes the rather lengthy Title 49 regulations; this guide is divided into sections on labeling and paper work, requirements for shipping specific compounds, emergency action techniques, and specific packaging instructions. There is a 50-pound maximum weight and 108 inches is the maximum girth plus length. In addition to the legally specified DOT labels, certain special labels and certification, as shown in this appendix are required on all hazardous materials shipments. UPS also supplies an "acknowledgment of delivery" form, included in this appendix, that would be useful in conducting audits. UPS employs a chemist on their headquarters staff who will determine acceptability, quantity, and packaging requirements for hazardous materials not mentioned in the UPS Guide. The Guide gives a useful list of telephone information numbers, also shown herein. According to UPS District Representatives, general UPS delivery schedules allow overnight deliveries within a 150-mile radius from the point

of shipment, second-day deliveries within 1,000 miles, third-day deliveries within 1,500 miles, and fourth-day deliveries within all of the Continental United States. UPS will not ship materials classified as poisons because they do accept foodstuffs and concurrent shipments of poisons and foodstuffs are forbidden by Federal law.

The applicable regulations for the mailing of hazardous materials through the U.S. Mail are contained in Section 124.28 of the Domestic Mail Manual. These regulations require the proper labeling as specified in Title 49 CFR plus packaging in a waterproof inner liner, absorbent cushioning material, and a sealed outer container. The overnight delivery available to certain cities through the Express Mail service would be applicable in the execution of a mailable audit materials program.

Depending on the size of the package, heat stability of audit materials, and destination, any of these modes of mailing could be selected. For smaller package sizes, Federal Express "Priority One" service guarantees overnight service and is the most costly. U.S. Mail Express Mail service with certification of receipt is mid-range in cost and also gives overnight service. UPS is least rapid and least costly for long-range shipment.

For comparison purposes, costs were computed for mailing a single, 1-pound package from Chapel Hill, North Carolina, to San Francisco, California. According to the Federal Express Service Guide, Priority One mailing of restricted materials would cost $30.84. Express mail from post office to addressee would cost $7.90. UPS shipment with acknowledgment of receipt would cost $1.55.

United Parcel Service

4477201

SHIPPER'S CERTIFICATION FOR HAZARDOUS MATERIALS

				STAMP YOUR UPS SHIPPER NUMBER ABOVE	
1.					
2.					
3.					
4.					
5.					
6.					
7.					
8.					
9.					
10.					
11.					
12.					
13.					
14.					

CERTIFICATION: This is to certify that the above named materials are properly classified, described, packaged, marked and labeled, and are in proper condition for transportation according to the applicable regulations of the Department of Transportation, Title 49, Part 100-199, Code of Federal Regulations, and Requirements of United Parcel Service

U1206 Rev. 1-78 (4084 BKS-11/75)

Shipper's Signature _____ *Date* __/__/__

TEAR HERE

SHIPPING PAPER – Abbreviations not permitted

UPS HAZARDOUS MATERIALS

Required Shipping Name of Material

D.O.T. Classification

Weight or Volume

Type D.O.T. Label Required

SHIPPER: Use ball point pen and press firmly when preparing this form.

LIFT ONE COPY OF FORM AND DETACH HERE

INSTRUCTIONS

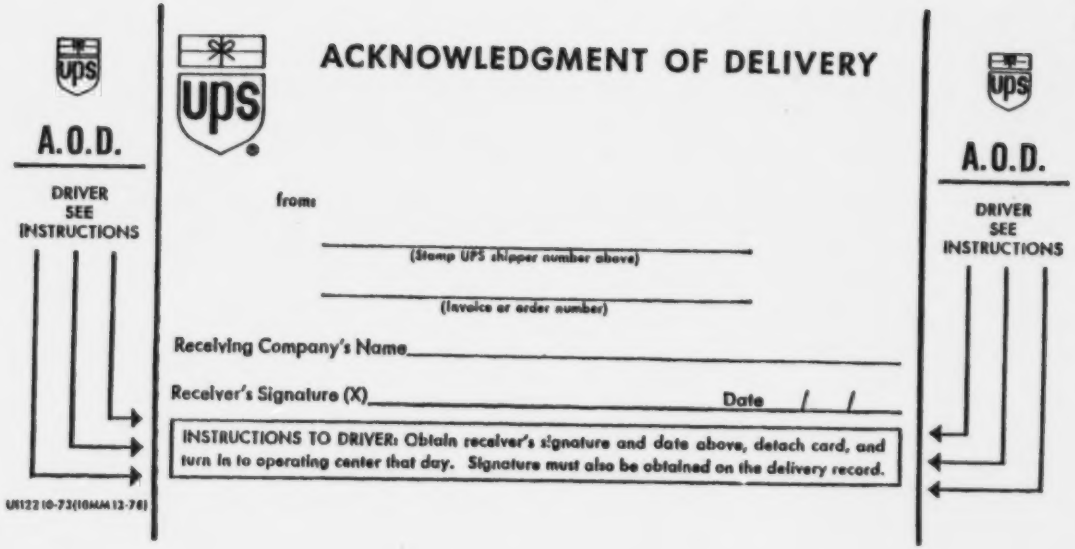
IN LOADING

Remove one copy of form and insert in Hazardous Materials envelope provided.

IN TRANSIT

Driver must have Hazardous Materials envelope in vehicle cab during transit.

U1114 6-75 (2MM 6-75)



A.O.D.

DRIVER SEE INSTRUCTIONS

UB122 10-73(10MM 12-78)

ACKNOWLEDGMENT OF DELIVERY

from

(Stamp UPS shipper number above)

(Invoice or order number)

Receiving Company's Name

Receiver's Signature (X) Date / /

INSTRUCTIONS TO DRIVER: Obtain receiver's signature and date above, detach card, and turn in to operating center that day. Signature must also be obtained on the delivery record.

A.O.D.

DRIVER SEE INSTRUCTIONS

32

Bureau of Explosives, Association of American Railroads (Explosives)	202-293-4048
Chemtrec (MCA) Chemical Transportation Emergency Center	
(Chemicals & Pesticides)	800-424-9300
(Alaska and Hawaii)	202-483-7616
(District of Columbia)	202-483-7616
Communicative Disease Center (Etiologic Agents – Biomedical Material)	404-633-5313
Department of Transportation, D.O.T. Office of Hazardous Materials Operations	202-426-0656
National Response Center, NRC (Oil/Hazardous substances)	800-424-8002
U.S. Coast Guard	800-424-8802*
For reporting of major incidents only	202-426-1830

Other Telephone Numbers Concerned with Hazardous Materials (for use during normal business hours)

D.O.T. Information Center (questions on regulatory matters)	202-426-9280 or 426-9281
National Agricultural Chemical Association Pesticide information	513-961-4300
United Parcel Service National Customer Service	203-622-6000

*Toll free

PRIORITY ONE: OVERNIGHT AIR SERVICE

The following paragraphs contain the general rules and regulations under which Federal Express Corporation (the Carrier, FEC) is engaged in the air transportation of small packages, documents and restricted articles between selected cities in the United States.

SERVICES:

- **Priority One: Overnight Air Service** is a door-to-door service with packages scheduled to be delivered to the consignee's address by 12:00 noon on the following business day after pick-up by or tender to FEC. In conjunction with Priority One Service, the following special services are available upon request: Restricted Articles Service, Saturday Delivery Service and Out of Area Service (see appropriate sections under "All Services" on pages 20-22).
- **Restricted Articles Service** is designed to provide special care and handling of certain materials defined as "hazardous for transportation purposes" by the Department of Transportation. Packages are scheduled to be delivered to the consignee's address by 12:00 noon on the following business day after pick-up by or tender to FEC.

Saturday Delivery Service and **Out of Area Service** are discussed in the appropriate sections under "All Services".

RATES:

Priority One: Overnight Air Service rates are calculated on a "per package" basis with Pick-up and Delivery Services included in the rate. These rates apply to all "Overnight Services" (except Courier Pak) with a surcharge of $8.00 per package for Saturday Delivery Service.

The surcharge for Restricted Articles Service ranges from $3.00 to $6.00 per package and is based on the number of Priority One Restricted Articles packages tendered on a Federal Express Account Number on any given day. Surcharges less than $6.00 are only available when packages are tendered on a Federal Express Account Number. The Restricted Articles Surcharge Schedule follows:

Number of Priority One Restricted Articles packages	Surcharge Per Package
1-5	$6.00
6-10	5.50
11-20	5.00
21-30	4.50
31-40	4.00
41-50	3.50
Over 50	3.00

RESTRICTIONS:

Overnight Air Services (Priority One and Restricted Articles Services):

a. No single package may exceed 70 pounds in weight or 108 inches in length and girth combined.
(1) "Length" means the longest dimension of a package.
(2) "Girth" means the shortest circumference of a package.
(2 × width + 2 × height)

b. No package exceeding 54 inches in length will be accepted for transportation.

c. Packages over 70 inches in length and girth combined will be assessed a minimum charge of a 25 pound package.
("length and girth combined" = length + 2 × width + 2 × height).

d. Due to the incompatibility of the following commodities, Federal regulations restrict the carriage of any package bearing a "Poison" label together with any foodstuff, feed or other edible material intended for consumption by humans or animals (designated herein as "foodstuff"). Accordingly, FEC restricts carriage of these commodities to the following schedule:
(1) Poison-labeled packages may only be tendered on Mondays, Wednesdays and Fridays.
(2) Foodstuff (as defined above) may only be tendered on Tuesdays and Thursdays. Packages containing foodstuff must be appropriately labeled.

e. Packages will not be accepted under Priority One Service when tendered to FEC in a "Courier Pak Overnight Envelope™", "Courier Pak Overnight Box™", or "Courier Pak Overnight Tube™", except as provided in paragraph "d" of "Courier Pak Restrictions" on the opposite page.

f. No more than 300 pounds from one shipper to one consignee will be accepted daily, unless advance arrangements have been made with FEC.

g. "Bulk Shipment" means a single consignment of up to 99 identical Priority One packages (same weight, size and declared value) from one shipper at one time at one address, receipted for in one lot, and moving on one Federal Express Airbill to one consignee at one destination address. Rates are still calculated on a "per package" basis.

Note: Refer to the section "Shipments Not Acceptable" under "All Services" on page 20 for further restrictions.

(In addition to the above, the following restrictions are applicable to the services indicated):

Restricted Articles Service:

a. Restricted articles are those materials defined as "hazardous for transportation purposes" by the Department of Transportation and which are regulated by Title 49, Code of Federal Regulations (CFR).

b. All packages must be limited to the materials and quantities authorized for air transportation according to the latest revisions of Title 49 CFR. Shippers must identify, classify, package, mark, label and document each shipment in accordance with the provisions set forth in the latest revision of Title 49 CFR Parts 170-189.

c. Each restricted articles shipment must be accompanied by a minimum of two copies of a properly executed Shipper's Certification.

d. Cryogenic liquids (Air, Argon, Helium, Neon and Nitrogen in a liquid state) will only be accepted when packaged as indicated in 49 CFR 173.304 including pressure controlling valve requirements.

e. Carcinogens; Etiologic Agents; Fissile Class III Radioactive Materials; Radioactive Material, limited quantity, N.O.S. or Radioactive Devices N.O.S. will not be accepted without advance arrangements by the shipper with FEC.

f. Class B Explosives shipments will only be accepted for carriage on an airport-to-airport basis due to Government regulations. Since many FEC Station facilities are located "off-airport", Class B Explosives cannot be shipped to or from those Station facilities. Therefore, shippers must contact the FEC Restricted Articles Department in Memphis (toll-free) 800 238-5355, ext. 3853 prior to any shipment of Class B Explosives to determine if both origin and destination Stations are equipped to handle such shipments. In any event, packages will not be accepted unless shipper and consignee have made arrangements in advance to deliver and pick up such shipments to/from the local FEC Station.

g. Restricted Articles packages, excluding Class B Explosives, will be accepted to "Agent" cities or destinations outside of the FEC Service Area only when advance arrangements have been made with the FEC destination Station.

*Scheduled 12:00 noon delivery is available in all locations within the FEC Service Area with the exception of "Extended Area" or "Agent" cities listed in sections 11 and 12 of this Guide.

SECTION 8

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SERVICE INFORMATION

COURIER PAK® SERVICE

SERVICES:

COURIER PAK® is the system of delivery of documents or any time-sensitive material up to five (5) pounds throughout the Federal Express Service Area in specially-designed shipping containers supplied by Federal Express. Courier Paks are scheduled to be delivered to the consignee's address by 12:00 noon on the following business day after pick-up by or tender to FEC. In conjunction with Courier Pak Service, the following special services are available upon request: Saturday Delivery Service and Out of Area Service (see appropriate sections under "All Services" on pages 20-22).

Three types of Courier Pak Service are available:

a. **Courier Pak Overnight Envelope™** is a tear- and water-resistant envelope measuring 12"×15½" which holds up to two (2) pounds of documents or lightweight material.

b. **Courier Pak Overnight Box™** is a crush- and water-resistant carton measuring 12½"×16"×3" which holds up to five (5) pounds of time-sensitive material requiring a sturdy container.

c. **Courier Pak Overnight Tube™** is a crush- and water-resistant, "cylinder-like" container measuring 3"×38"×3" which holds up to five (5) pounds of time-sensitive items that require protection from folding, creasing or bending.

RATES:

COURIER PAK® Service rates, including Pick-up and Delivery Service, are as follows:

a. Overnight Envelope: $19.00 each
(up to two pounds of material)
b. Overnight Box: $24.00 each
(up to five pounds of material)
c. Overnight Tube: $24.00 each
(up to five pounds of material)

A surcharge of $8.00 per Courier Pak will be assessed for Saturday Delivery Service. Courier Pak rates include a declared value of $100.00 with higher declared value available at $.30 per each additional $100.00 up to a maximum of $2000.00 per Courier Pak.

RESTRICTIONS:

COURIER PAK® Service:

a. Shipments moving under Courier Pak Service must be tendered in a Courier Pak Overnight Envelope™, Overnight Box™ or Overnight Tube™ supplied by Federal Express.

b. Courier Pak Service is provided subject to the restrictions printed on each Courier Pak container.

c. When utilizing the Courier Pak Services of Federal Express, FEC customers are exempt from the Private Express Statutes pursuant to 39 CFR 320.6(c).

d. The gross weight of any one Courier Pak (including the container) is limited to two (2) pounds for the Overnight Envelope and six (6) pounds for the Overnight Box and Overnight Tube, and of a size and shape which fits in the container without danger of damaging the container. The shipper is responsible for providing any additional packaging required to ensure safe handling of the Courier Pak and protection of its contents. If the gross weight of a Courier Pak exceeds these limitations, the current Priority One rate will be assessed for its actual gross weight in lieu of the regular Courier Pak charge.

e. Restricted Articles Service is not available in conjunction with Courier Pak Service.

f. Collect Courier Paks will be accepted as per the "Collect Shipments" section under "All Services".

g. No more than one Courier Pak may be shipped and no more than one service may be indicated on a single Airbill.

Note: Refer to the section, "Shipments Not Acceptable" under "All Services" on page 20 for further restrictions.

STANDARD AIR SERVICE

SERVICE:

Standard Air Service is a door-to-door service with shipments scheduled to be delivered to the consignee's address on the second business day after pick-up by or tender to FEC. Out of Area Service is available (see appropriate section under "All Services" on page 20.)

RATES:

Standard Air Service rates are calculated on a per shipment basis with Pick-up and Delivery Services included in the rate.

A surcharge of from $3.00 to $6.00 per shipment will be assessed for those radioactive shipments and commodities classsified as Other Regulated Materials (ORM) in Title 49, Code of Federal Regulations (including shipments containing Dry Ice). This surcharge is based on the number of Standard Air Restricted Articles shipments tendered on a Federal Express Account Number on any given day. The Restricted Articles Surcharge follows:

Number of Standard Air Restricted Articles Shipments	Surcharge Per Shipment
1-5	$6.00
6-10	5.50
11-20	5.00
21-30	4.50
31-40	4.00
41-50	3.50
Over 50	3.00

As used herein, the term "shipment" means a single consignment of one or more packages from one shipper at one time at one address, receipted for in one lot, and moving on one Federal Express Airbill to one consignee at one destination address.

RESTRICTIONS:

Standard Air Service:

a. The maximum acceptable weight per shipment is 300 pounds.

b. No single package as part of a shipment may exceed 70 pounds in weight or 108 inches in length and girth combined.

(1) "Length" means the longest dimension of a package.
(2) "Girth" means the shortest circumference of a package.
(2 × width + 2 × height).

c. No package exceeding 54 inches in length will be accepted for transportation.

d. If any single package within a shipment exceeds 70 inches in length and girth combined, the entire shipment must be rated as "oversize" and the appropriate box checked on the Airbill. A surcharge of $15.00 will be added to the applicable shipment charge. ("length and girth combined" = length + 2 × width + 2 × height)

e. Only those restricted commodities classified as Radioactive or Other Regulated Materials (ORM) in Title 49 CFR (including shipments containing Dry Ice) will be accepted. In these cases, all Restricted Articles restrictions under "Overnight Air Services" will apply.

f. Firearms will not be accepted.

g. No more than 300 pounds from one shipper to one consignee will be accepted daily, unless advance arrangements have been made with FEC.

Note: Refer to the section "Shipments Not Acceptable" under "All Services" on page 20 for further restrictions.

The "Federal Express Service Guide" is published quarterly. Changes and additions to this Guide are printed in blue for emphasis.

SECTION **8**

APPENDIX B
HERL DATA SYSTEM FOR IN VITRO TESTS

HERL INTERIM IN VITRO SYSTEM

USER FORMS COMPLETION INSTRUCTIONS

Health Effects Research Laboratory
Data Management Branch, Biometry Division
Research Triangle Park, North Carolina 27711

Prepared By: Carol C. Evans

April 1980

INTRODUCTION

The Interim In Vitro System has been developed by the Health Effects Research Laboratory of EPA in Research Triangle Park, North Carolina, for the purpose of systematizing the collection of experimental results of IN VITRO tests run by the Genetic Toxicology Division and their contractors. A series of computer programs has been written to allow for verification of experimental results, statistical analysis of data, and reports of raw data collected, etc. This system is a scaled-down version of the IN VITRO system developed by the National Cancer Institute under the direction of Ms. Mary S. Linhart. The basic concept of their forms design, forms completion instruction, and coding conventions has been retained by HERL.

Experimental results will be collected on forms, keypunched, and input into the IN VITRO network of computer programs. Currently, the system is designed to handle only results from the AMES (plate incorporation) test. It will be modified in the future to handle results from a variety of IN VITRO tests.

All data is to be entered on forms and will be filled out by the technicians conducting the experiments. Blank forms can be obtained by xeroxing or by contacting Ms. Dorothy Denning or Ms. Bonita Hodges, Data Preparation and Control Section, MD-55, EPA, Research Triangle Park, North Carolina 27711. When using the 3-part self-carbon forms, submit the top white sheet to Ms. Denning. The other two parts may be retained by the testing lab for their own records. When a contractor submits keypunched data (cards or tape), he should send one copy (any color) of the forms also. Any questions concerning how to complete a form or submit data may be addressed to Ms. Carol Evans (919-541-2567 or FTS 629-2567).

GENERAL INSTRUCTIONS

At the present time, there are two user forms; the results form (and its continuation sheet) and the sample ID form. A third form, the activation batch preparation form, is being developed.

All written entries should be clear and dark.

Code numbers are required as entries for many items in order to obtain uniform data. For user convenience, the codes have been printed on the forms when possible. Some items require referring to the Code Tables in this manual for completion. If the Code Tables do not contain an appropriate code for the response, use the "OTHER" code and write the response in the REMARKS section of the form. Use the circled item code (a letter in a circle) to identify the item to which the remarks pertain.

On every form you complete, please note your initials in the blocks in the "Forms Completion" area at the bottom right of the sheet. It is a good practice to note the date nearby.

HERL IN VITRO RESULTS FORM

KEY ITEMS

The items on the top line of the form (Research Lab ID through Microorganism Strain) uniquely identify a test. They must be completed. (Please note the exception for Microorganism Batch Number and the special instructions for Activation Batch at this time.)

TOP LINE

Update Code - Do not complete. This item is for use by HERL data processing personnel only.

Research Lab ID - Enter the complete 4-letter code which identifies your organization. This identifies the group that is conducting the experiment. This code may be obtained from the Genetic Toxicology Division, HERL/RTP.

Experiment Date - Enter the date the experiment was started. Use the standard month-day-year notation.

Test Sample Identification - Enter the full 10-character sample identification number which identifies the agent being tested. This number was assigned by EPA. It consists of a 4-letter sample preparation lab code followed by a 2-digit year of preparation followed by a 4-digit number.

Activation Batch - If the results reported on this form refer to a test done without activation, enter a '-' left justified (Column 25). If the results reported on this form refer to a test done with activation, enter a '+' in Column 25. Leave the other columns (26 through 30) blank. If a test is done with and without activation, two forms must be completed, separating the test results accordingly.

Instead of denoting a test done with activation by a '+' in Column 25, you may enter an activation preparation batch identifier in positions 25 through 30. It is RECOMMENDED that the first three characters be used as follows:

1st character	R	= rat
	M	= mouse
	H	= hamster
2nd character	L	= liver
	U	= lung
	K	= kidney
	T	= testes
3rd character	I	= induced
	N	= non-induced

The remaining three characters may contain a unique numeric identifier. Thus, RLI001 is first Rat Liver Induced Activation Preparation for a testing laboratory (research lab ID). RLI002 is the second Rat Liver Induced Activation Preparation, etc.

Test Type - Enter the appropriate test type code from Table 10.

Microorganism Strain - Enter the appropriate strain abbreviation from Table 8. If the strain abbreviation is less than 6 characters, enter it left-justified starting in Column 33.

Microorganism Strain Batch - not accepted at this time.

SECOND LINE

Remarks Made - If you made any remarks concerning the circumstances of this test in the REMARKS area at the bottom of the form, enter a code of "1" for "yes". If no remarks were made, enter a "2" or leave blank for "no."

Phenocopy Check Conclusion - Enter the appropriate code from Table 13 to identify the conclusion concerning the results of restreaking (or replicate plating) when colonies were restreaked to check for phenotypic reversion.

Sterility S-9 Mix - Using the codes that appear on the form, enter the results of checking the sterility of the S-9 mixture. "CONTAM" is the space-saving abbreviation for "CONTAMINATED."

Activation Mixture Per Plate - Enter (right-justified) the amount of activation mixture plated on each plate. The amount must be reported in microliters. If activation mixture was not used, leave this item blank.

Pre-incubation Time - If the test type indicated pre-incubation, enter the duration of incubation in minutes right-justified. Otherwise, leave this item blank.

Pre-incubation Temperature - If the test type indicated pre-incubation, enter the temperature of incubation in degrees centigrade. Otherwise, leave it blank.

Technician - Enter the initials of the technician conducting the test. Always supply the same three initials for a technician. If more than one technician has the same initials, assign a unique set of initials to each one (e.g., vary the middle initial to differentiate). If a technician has no middle initial, make one up.

On some of the forms, space has been left for the user to write in information about elements of the experiment: animal, organic, inducer, microorganism. This space is provided for user convenience, and the data will not be entered into the computer master files.

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SUBSEQUENT LINES (Card Codes B through Y)

Subsequent lines on the form are to be used for reporting the plate counts for the positive and negative control(s) and the various dose levels of the test agent. A maximum of 24 doses can be reported by using the continuation form. The form allows for a maximum of five replicate plates at each dose. THIS IS IMPORTANT: Enter information in the order described below and do not skip lines.

Solvent/Positive - When reporting solvent (negative control) or positive control plate counts, enter the appropriate code from Table 11 denoting the substance used as the control agent. For a negative control entry, if no solvent was used, enter a code of "50." If more than one positive or negative control substance was used, enter the appropriate code and data on subsequent lines. Enter all negatives on consecutive lines first, then all positives. Leave this area blank when reporting results for test agent (sample ID doses).

Even though the same positive and negative controls were used for a number of tests, the control plate counts must be repeated on each form.

Units of Concentration - Enter the code as shown on the form to identify the units in which the stock concentration is reported. Only µg/ml or mg/ml are valid. This field may also be used to identify the units (µg/plate) of a dose which was pre-computed by the technician. In this case, report the pre-computed dose in the stock concentration field, leaving amount per plate blank.

Stock Concentration - Enter right-justified the stock concentration in either milligrams per milliliter or micrograms per milliliter. Leading zeros are not required. If the dose is in microliters, (as with a liquid sample), this item may be left blank. When reporting the plate counts for a negative control denoted as a "50," this item may be left blank also.

Amount Per Plate - Enter in microliters, right-justified, the amount of liquid plated on an individual plate. This field may be left blank when reporting plate counts for a general negative control (positive/solvent = "50"). If the actual dose level is in microliters, enter it here.

NOTE: It is desirable to report both stock concentration and amount per plate whenever possible. The computer programs will then calculate the actual dose in micrograms per plate for you.

Plate Counts A through E - Enter the number of colonies actually counted on each plate. Enter the number right-justified; leading zeros are not required. If a plate was counted at zero, enter a "0"; do not leave it blank. Other legitimate entries are "TNTC" for "too numerous to count;" "NATC" for "not able to count;" or T* (left-justified) for "toxic." If less than five plates were made and

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counted, leave non-used plate counts blank. Enter plate counts starting at A, then proceed to B, etc.

BG (Background Code) - Enter the code from Table 12 which designates the appearance of the background lawn of cells for the corresponding plate. If this item is left blank, it is assumed that the background lawn was observed to be normal.

REMARKS

Enter here any remarks you wish to make concerning the circumstances of an experiment. If the remark pertains only to a particular dose level entry, enter the card code ("B" through "Y") of the line on which the dose was reported. Remarks pertaining to information reported on the continuation form should be reported here also. If you run out of space, attach a blank sheet of paper and continue. Keep remarks clear and concise when possible. They will also be keypunched.

CONTINUATION FORM

The continuation form should be used when the nine dose lines on the original form will not accommodate all the controls and doses for a particular test. The top line of the continuation sheet should be completed to match the top line of the first results form. The two sheets should then be stapled together and submitted as a unit.

ERROR CORRECTIONS

Occasionally, changes will be required on forms already submitted: correction of a transcription mistake, adding a dose line for a test which was accidentally omitted, or removing blocks of data that are invalidated for some reason.

This can be done in the following manner:

1. Make a xerox of the form that contains erroneous data.

2. Using a color of ink which stands out (*such as red), line out the erroneous data. Then show the correct values just above it.

3. You may wish to clarify or further describe the correction or addition being made with a note in the REMARKS area.

4. Clearly mark "UPDATE" at the top of the form.

or

5. If you can not locate the form on which the incorrect data was previously submitted, fill out a new form. Clearly mark it "UPDATE" at the top.

6. You must always complete the top two lines to identify the test. If the change or addition you wish to make involves items in the top line, it

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is preferable to use the first method of correction. If this is not possible, note the original contents of the top line in the REMARKS area.

7. If the change involves one (or more) of the dose lines, fill out completely all items starting with the card code "B:" line. Then, in the REMARKS area, note whether the dose line you have entered is to be "DELETED," "ADDED," or be a "REPLACEMENT" for a previously reported dose line. If the line you enter is a replacement for a previously reported dose line, identify the line you wish to replace in the REMARKS area.

An alternate method may also be used:

If you have received a report of test results that have been entered into the computer files, you may use it to note corrections in a clearly visible color of ink. Mail a copy of the corrected report sheets to Ms. Denning. Currently, two report formats exist. The plate count report lists results on a test-by-test basis but does not include all items that were entered on the results form. The quality assurance listing dumps test results from the master computer file in a format similar to the original results form.

SAMPLE IDENTIFICATION FORM

This form provides for registry of a chemical test sample code as a valid test agent. It also allows the user to specify a chemical name or other descriptive title (up to 41 characters) to be used in report headings. Use of a sample ID Code on a results form which has not been registered via this form will delay the entry of the test results into the computer files.

The sample identifier is assigned by the EPA lab or analysis group that furnishes a test agent sample to the testing laboratory. It is formed as follows:

Pos. 1 - 4	Valid sample preparation lab code from tables
5 - 6	Preparation year
7 - 10	Unique number

HERL INTERIM IN VITRO SYSTEM

TABLES

TABLE 8. MICROORGANISM STRAIN CODES

Ames Strains:	TA1535	
	TA1537	
	TA1538	
	TA100	
	TA98	
	TA90	
	TA92	
	LT2	
Ames Strains Used for 8AG Resistance:		8A1535
		8A1537
		8A1538
		8A100
		8A98
Skopek-Thilly Strains:	TM35	
	TM677	
Nitroreductase Deficient Strains:		1535ND
		1537ND
		1538ND
		98ND
		100ND
E. Coli Strains:	P3478	
	W3110	

TABLE 10

Test Type Codes

Standard Plate Incorporation	01
Spot Test	02
Plate Test - Gas Chamber	03
Plate Test - Vapor From Spot on Lid	04
Plate Test - Pre-Incubation	05
Suspension Test	06
Suspension Test Exposed to Gas	07
Suspension Test - Gas Bubbled	08
DNA Repair Spot Plate Test	09
DNA Repair Suspension Test	10
Survival Data - Pre-Incubation	15
Survival Data - Suspension Test	16
Other	99

TABLE 11

Solvent Codes	
Negative (no solvent)	50
Distilled Water	51
Normal Saline	52
K Phosphate Buffer 0.050-0 10M ph 7.0	53
Dimethylsulfoxide	54
100 percent Ethanol	55
Methanol	56
Acetone	57
Other Solvent	99
Positive Codes	
Naazide	01
9-AA	02
2-NF	03
2-AA, 2A, 2-Aminoanthracine	04
MNNG	05
AF-2 (Aflatoxin)	06
2-Aminofluorene (2AF)	07
DMBA	08
Other Positive	09

TABLE 12

BACKGROUND CODES

Normal	1, blank
Partially Clear	2
Clear	3
Contaminated	4
Precipitation and Normal	5
Precipitation and Partially Clear	6
Precipitation and Clear	7
Small Colonies	8
Other	9
Heavy	A

TABLE 13

PHENOCOPY CHECK
CONCLUSION CODES

True Mutants	1
Pheno Copies	2
Mixed Population	3
Questionable	4
Other	9

HERL IN VITRO RESULTS FORM

1	2–4	5–8	9–14	15–18	19–20	21–24	25–30	31–32	33–38	39-42
UPDATE CODE	SYSTEM ID (I V R)	RESEARCH LAB ID	EXPERIMENT DATE (MO DA YR)	TEST SAMPLE IDENTIFICATION: LAB	YR	NUMBER	ACTIVATION BATCH	TEST TYPE (Table 10)	MICROORGANISM: STRAIN	BATCH NO

ANIMAL / ORGAN / INDUCER / MICROORGANISM	(A) 64	(B) 65	(C) 66	(D) 67–70	(E) 71	(F) 72–73	74–75	(G) 76–78	80
	REMARKS MADE? YES=1	PHENOCOPY CHECK CONCLUSION (Table 13)	STERILITY S-9 MIX 1–NOT CONTAM 2–CONTAM 3–NOT CHECKED	ACTIVATION MIXTURE PER PLATE (µl)	SAMPLE STERILITY CHECK 1–NOT CONTAM 2–CONTAM 3–NOT CHECKED	TIME(MIN) PRE-INCUBATION	TEMP(CENT)	TECHNICIAN	CARD CODE

	DOSE LEVEL			PLATE A		PLATE B		PLATE C		PLATE D		PLATE E		
(H) SOLVENT POSITIVE (Table 11)	(I) UNITS OF CONCENTRATION Blank = mg/ml 2= ug/ml	(J) STOCK CONCENTRATION	(K) AMT. PER PLATE (µl)	(L) COUNT	(M) BG	(N) COUNT	(O) BG	(P) COUNT	(Q) BG	(R) COUNT	(S) BG	(T) COUNT	(U) BG	CARD CODE
43 44	45	46–50	51–54	55–58	59	60–63	64	65–68	69	70–73	74	75–78	79	80
SOL														B
POS														C
														D
														E
														F
														G
														H
														I
														J

REMARKS: INDICATE ITEM CODE AND CARD CODE

SAMPLE

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FORMS COMPLETION	
IN	LS

HERL IN VITRO RESULTS CONTINUATION FORM

1	2–4	5–8	9–14	15–18 – 19–20 – 21–24	25–30	31–32	33–38	39–42
UPDATE CODE	I V R SYSTEM ID	RESEARCH LAB ID	MO DA YR EXPERIMENT DATE	LAB YR NUMBER TEST SAMPLE IDENTIFICATION	ACTIVATION BATCH	TEST TYPE (Table 10)	STRAIN	BATCH NO
							MICROORGANISM	

		DOSE LEVEL		PLATE A		PLATE B		PLATE C		PLATE D		PLATE E		
Ⓗ SOLVENT POSITIVE (Table 11)	Ⓘ UNITS OF CONCENTRATION Blank= mg/ml 2= ug/ml	Ⓙ STOCK CONCENTRATION	Ⓚ AMT. PER PLATE (µl)	Ⓛ COUNT	Ⓜ BG	Ⓝ COUNT	Ⓞ BG	Ⓟ COUNT	Ⓠ BG	Ⓡ COUNT	Ⓢ BG	Ⓣ COUNT	Ⓤ BG	CARD CODE
43–44	45	46–50	51–54	55–58	59	60–63	64	65–68	69	70–73	74	75–78	79	80
														K
														L
														M
														N
														O
														P
														Q
														R
														S
														T
														U
														V
														W
				SAMPLE										X
														Y

HERL IN VITRO SYSTEM
SAMPLE IDENTIFICATION FORM (INTERIM)

SAMPLE

SYSTEM ID: I V S (2–4)

SAMPLE ID LAB (5–8)	YR (9–10)	NO (11–14)	SAMPLE DESCRIPTION (15–56)

APPENDIX C
SUMMARY QUESTIONNAIRE FOR AMES/*SALMONELLA* PROTOCOL

INSTRUCTIONS

After each question, please place an "X" in the box with the appropriate answer for that question. Or, if appropriate, fill in the blank after the question.

(Ignore small numbers in the right margin of the questionnaire. These are keypunching codes.)

1. **Is the Ames Protocol (1975), as modified by de Serres and Shelby (1979), followed?**

   ☐ Yes ☐ No 1

2. **How is the initial toxicity range determined for use in mutagenicity testing?**

   ☐ Qualitative testing ☐ Quantitative testing 2

3. **What type of test is used?**

   ☐ Pour 3

   ☐ Spot

   ☐ Well

   ☐ Suspension

4. **Is preincubation used?**

   ☐ Yes ☐ No 4

5. **What animal was used in preparing mammalian microsomal enzyme systems?**

   ☐ Rat 5

   ☐ Mouse

   ☐ Hamster

   ☐ Other *(Specify)* _____

   5a. **What animal weight range is allowed?** _____ g to _____ g 6-9, 10-12

6. **How many dosage levels are normally used?**

   _____ levels 13-14

7. **How many replicate plates are used routinely for each dose?**

   _____ replicate plates/dose [redacted] 15-16

8. **What range is allowed in number of viable cells/plate in the mutagenicity test?**

   _____ to _____ cells X 10^8 17-20, 21-24

9a. **Was S-9 induced?**

25

☐ Yes ☐ No

If the answer to 11a was yes, please answer question 11b; otherwise, proceed to question 12.

9b. **Was Aroclor 1254 compound used?**

☐ Yes ☐ No 26

10. **What is the maximum allowable storage time and storage temperature for S-9?**

(a) _____ days 27-29

(b) _____ °C 30-32

11. **What is the incubation time and temperature routinely used for the agar plates?**

(a) _____ hours 33-35

(b) _____ °C 36-38

12. **What is the laboratory's spontaneous background range for the following bacterial strains?**

(Leave spaces blank if a strain is not used.)

a. **Without metabolic activation**

	Low	High	Mean	Standard deviation	No. of experiments	
TA 1535						39-41, 42-44, 45-47, 48-50, 51-57
TA 1537						58-60, 61-63, 64-66, 67-69, 70-76
TA 1538						1-3, 4-6, 7-9, 10-12, 13-20 (2)
TA 98						21-23, 24-26, 27-29, 30-32, 33-40
TA 100						41-43, 44-46, 47-49, 50-52, 53-60

b. **With metabolic activation**

	Low	High	Mean	Standard deviation	No. of experiments	
TA 1535						61-63, 64-66, 67-69, 70-72, 73-80
TA 1537						1-3, 4-6, 7-9, 10-12, 13-20 (3)
TA 1538						21-23, 24-26, 27-29, 30-32, 33-40
TA 98						41-43, 44-46, 47-49, 50-52, 53-60
TA 100						61-63, 64-66, 67-69, 70-72, 73-80

c. **Period of time over which values compiled for these ranges** _____ months 1 (4)

13. **Is entire experiment for each chemical with each strain routinely repeated on a different day from the initial testing?**

☐ Yes ☐ No 2

14a. Are any outlier rejection procedures used?

☐ Yes ☐ No 3

14b. If yes, please describe.

4

15a. How was a positive mutagenic response determined?

☐ Quantitative ☐ Qualitative 5

15b. Please describe criteria used to determine mutagenicity:

6

16. How many doses are routinely run in an experiment?

_____ doses 7-9

17. Were enzyme levels measured for S-9 activity before use?

☐ Yes ☐ No 10

18. Was S-9 evaluated against known positive controls (such as 7, 12-Dimethylbenzanthracene [DMBA])?

☐ Yes ☐ No 11

19a. Are bacterial strains checked for markers (ampicillin resistance, cell wall mutation, UV sensitivity, and histidine sensitivity)?

☐ Yes ☐ No 12

If the answer to 19a is yes, please continue; otherwise, go to question 20.

19b. How often are bacterial strains checked for markers?

_____ days 13-15

20. Were revertants checked by replating (phenocopy check)?

☐ Yes ☐ No 16

21a. How were plates counted?

☐ By hand ☐ By machine 17

21b. Describe the machine.

18

22. Were all test components tested separately for sterility?

☐ Yes ☐ No 19

References

Ames, B. N., J. McCann, and E. Yamasaki, Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test, *Mutation Res.*, 31:347-364, 1975.

De Serres, F. J., and M. Shelby, Recommendations on data production and analysis using the Salmonella/microsome mutagenicity assay, *Mutation Res.*, 64:159-165, 1979.

COMMENTS OR ADDITIONAL INFORMATION: *(Please use additional sheet if necessary)*

APPENDIX D
DATA INTERPRETATION MODELS

SOME NEGATIVE BINOMIAL MODELS FOR THE DISTRIBUTION OF REVERTANTS IN THE AMES TEST

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SECTION 1

INTRODUCTION

Mutagenicity, as measured by the Ames test, is highly correlated with carcinogenicity (Ames et al., 1975). Further, the Ames test is considerably more economical in time and materials than either an epidemiologic study or a whole-life animal study. For these reasons, the Ames test has emerged as an important and widely used component in the overall program of screening for potential carcinogens. Despite the fact that thousands of Ames tests are performed annually, surprisingly little attention has been paid to the proper statistical analysis of such data (de Serres and Shelby, 1979). A notable exception is the recent contribution of Stead et al. (1980), which models the number of revertants per plate at dose x as a Poisson random variable with mean

$$m(x) = (\beta_0 + \beta_1 x^{\beta_2}) e^{-\beta_3 x} . \qquad (1)$$

The mean number m(x) of revertants at dose x is thus regarded as a power function of dose attenuated by an exponentially decreasing function of dose to account for toxicity. A variety of data sets were analyzed with this model by Stead et al. (1980), demonstrating the possibility of systematic evaluation of Salmonella plate assay data within the framework of a formal statistical model.

The Poisson model may not be appropriate for all situations. Grafe and Vollmar (1978) observed that the variance of replicate plate counts tends to exceed the mean at mean levels above 150 revertants per plate. A Poisson model, which necessarily entails variance equal to mean, could not be strictly correct in such a case. This variance-greater-than-mean phenomenon has also appeared in RTI data (Figure 2).

This paper presents a plausible model that generalizes the approach of Stead et al. (1980), and accommodates variance-greater-than-mean among repli-

cate plates. The model is based on the negative binomial probability distribution and behaves like the Poisson model (1) of Stead et al. (1980) for smaller mean levels of revertants. The model attributes the excess variance to fluctuations in the number of bacteria initially plated. Margolin (1979) has asserted that the negative binomial distribution is more appropriate than the Poisson distribution for Ames test data. A similar application of the negative binomial model was employed by Greenwood and Yule (1920), who used it to describe numbers of accidents in World War I British munitions factories, and reported that the fit was superior to that provided by the Poisson distribution. The derivation here is somewhat analogous to theirs, which assumed that each worker's number of accidents was Poisson distributed with a mean specific to that worker, and that the different means among the workers were independent realizations of a gamma random variable.

A derivation of the negative binomial model for Salmonella plate assay data is presented in Section 2. Several commonly arising dose response curves for Ames test data are identified in Section 3, and biologically based parametrizations are offered. Saturation of bacteria cells with compound and toxic effects of compound are among the available options. The application of this package of models to six bioassay data sets is discussed in Section 4. The last two examples illustrate the value of auxiliary toxicity experiments in interpreting mutagenicity data; the toxicity experiments are done with excess histidine to promote continued cell division. Section 5 presents a summary of the work, a discussion of advantages, and some remaining problems. Computational details and a copy of the computer program are in the Appendix.

SECTION 2

DEVELOPMENT OF THE PROBABILITY MODEL

For a given plate at dose x of the test compound in an Ames assay, let N denote the unknown number of bacteria alive immediately after plating and available to revert back to the wild type or to be killed by toxic effects. Bacteria of a particular strain are genetically identical, so it is reasonable to assume that any two of them have the same probability p(x) of surviving the toxic effects of the agent and undergoing the reverse mutation. Assuming that the bacteria revert independently of one another, the probability of k revertants on the plate may be taken as the probability of k heads in N tosses of a coin with chances p(x) of landing heads,

$$P\ [k \text{ revertants}] = \binom{N}{k} p(x)^k\ (1-p(x))^{N-k} \quad . \qquad (2)$$

The number N varies from plate to plate according to the replicability of the plating procedure and the random mechanism governing which bacteria survive the plating. N is, for all practical purposes, a random number. The mean E(N) of N is (hopefully) the laboratory's target value for the number plated, and the variance var(N) of N is an indication of the laboratory's control over N. It is plausible that a major component of the aforementioned excess variance over the mean in replicate plate counts is due to variation in the number N of bacteria surviving the plating. The inherent randomness of N can be taken into account by selecting a family of plausible probability distributions for N and averaging the distribution of k revertants with respect to the generic member of the family. The resulting average probability distribution depends on the parameters that characterize the distribution of N. It is desirable that the family of distributions be computationally convenient and represent a reasonable variety of distributional shapes that are scientifically plausible for the true distribution of N.

We pass first to a Poisson approximant for (1), letting $p = p(x)$.

$$P\ [k \text{ revertants}] = (Np)^k\ [\exp(-Np)]/k! \qquad (3)$$

Viewing (3) as a function of N suggests the gamma family will meet the computational simplicity criterion. The gamma family of probability densities is a two-parameter family having the flexibility to represent a wide variety of distributional shapes, including exponential, chi-square, and (approximately) normal. The gamma probability density for N with shape parameter $\underline{a} > 0$ and scale parameter $\underline{b} > 0$ is

$$f(N \mid a,b) = cN^{a-1} \exp(-N/b) \quad , \qquad (4)$$

where $c = 1/(\Gamma(a)b^a)$ and $0 < N < \infty$. Note that the distribution of N has been approximated by that of a continuous random variable. The mean and variance of the gamma distribution are ab and ab^2, respectively.

Equation (3) gives an approximate distribution for the conditional probability of k revertants, given that N bacteria survive the plating. Equation (4) specifies the distribution of N, given the shape and scale parameter. The product of equations (3) and (4) yields the joint distribution of K and N. Integrating this joint distribution over the range of N ($0 < N < \infty$) results in the unconditional average probability of k revertants,

$$P[k \text{ revertants}] = \binom{a+k-1}{k} \left(\frac{bp}{bp+1}\right)^k \left(\frac{1}{bp+1}\right)^a \quad . \qquad (5)$$

Equation (5) specifies a <u>negative binomial</u> probability distribution for the number k of revertants. Let $Q = bp/(bp + 1)$ and $P = 1-Q = 1/(bp + 1)$. Then the mean number of revertants is $m = E(K) = aQ/P = abp$. This is the same as $[E(N)]p$ = (mean of N) · p, which is what would be expected intuitively. The variance of the number of revertants is $v = \text{var}(k) = aQ/P^2 = abp(bp + 1) > abp = E(K)$. Letting $c = 1/a$, the variance $v(x)$ of the number of revertants at dose x becomes

$$v(x) = m(x)\ [(m(x)/a) + 1] = m(x)\ [cm(x) + 1] \quad . \qquad (6)$$

Thus, the derived negative binomial probability distribution for the number of revertants has the desired property of variance larger than mean. The Poisson distribution is a limiting case, corresponding to a distribution for N with zero variance.

SECTION 3

PARAMETRIZATION OF THE MEAN NUMBER OF REVERTANTS

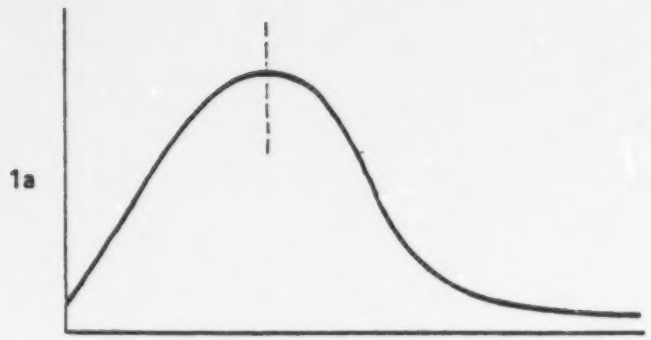
The issue of how to represent the probability of a reverse mutation can be addressed by reasoning analogous to that underlying multistage theories of carcinogenesis (Whittemore and Keller, 1978). This leads to the functional form $p(x) = 1 - \exp[-g(x)]$ for the probability of a reverse mutation at dose x; $g(x)$ is a nonnegative polynomial. According to Ames (1979), most Ames test results exhibit an initially linear dose response relation. Also, a linear dose response relation corresponds to the requirement of a single event for reverse mutation. Specializing g to a linear function and incorporating toxicity effects in the fashion of Stead et al. (1980) leads to $p(x) = 1 - \exp[-(r + sx)] \exp(-Tx)$. Because reverse mutations are usually quite rare, even at high doses of a mutagen, a first order Taylor approximation to the first multiplicand is reasonable and leads to $p(x) = (r + sx) \exp(-Tx)$. This approximation would be questionable in cases where large numbers of revertants are obtained despite extremely high toxicity. Preliminary toxicity experiments, if feasible, can aid in designing the mutagenicity experiments to avoid dose ranges yielding high toxicity. The mean number $m(x) = abp(x)$ of revertants at dose x can be parametrized similarly by absorbing the (ab) term in both the slope and intercept to obtain

$$m(x) = (B + Sx) \exp(-Tx) \quad , \tag{7}$$

which is a special case of the parametrization of Stead et al. (1980). The parameters B, S, and T are all constrained to be nonnegative.

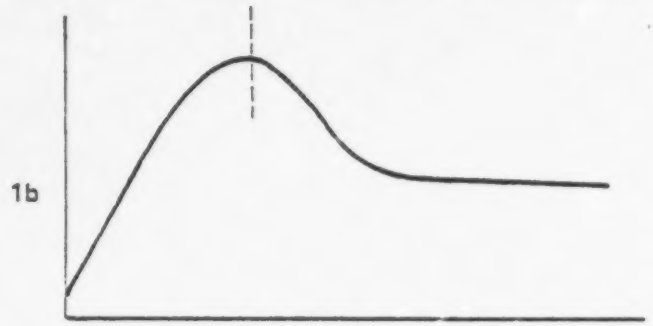
In the presence of toxicity ($T > 0$), the model specified by (7) is approximately linear in the range where Tx is small, increases to its maximum value, and then decreases to 0 as the dose x approaches infinity (Figure 1a).

For some Salmonella plate assay data sets, the underlying dose response curve appears to level off between the maximum value and zero (Figure 1b), or



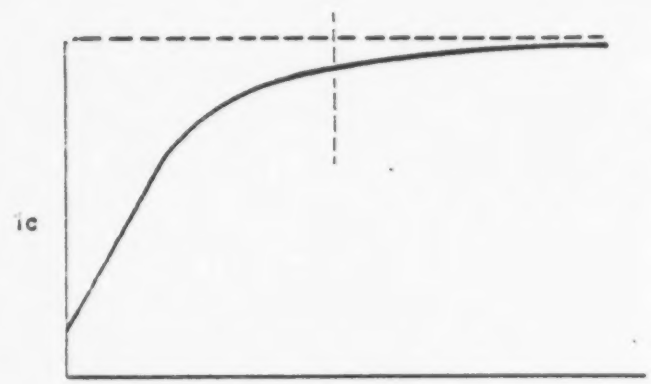
1a

$$m_2(x) = (B + Sx)e^{-Tx}$$



1b

$$m_2(x) = [B + V(1 - e^{-Sx/V})]e^{-T(1 - e^{-Sx/V})}$$



1c

$$m_3(x) = [B + V(1 - e^{-Sx/V})]$$

Figure 1. Some special cases of the general form

$$m(x) = E(N)\ P(s|x)\ P(r|s;x)$$

where

- $E(N)$ = the mean number of cells surviving the initial plating, available to reverse mutate or die of toxicity of the compound,
- $P(s|x)$ = the conditional probability of surviving the toxic effects of a dose x of the compound, given survival of the initial plating, and
- $P(r|s;x)$ = the conditional probability of a reverse mutation at dose x, given survival of the initial plating and the toxicity of the compound,
- B = the background mean number of revertants,
- S = the initial slope, and
- T = a toxicity rate constant.

increase to a horizontal asymptote (Figure 1c). This behavior may be explained through the concept of metabolized dose (as opposed to administered dose). That is, there may be a saturation limit to the association between compound and (bacterial) cell. If a model such as that specified by equation (7) is viewed as a function of metabolized dose, then the corresponding curve relating mutagenicity to administered dose could theoretically level off between the maximum and zero. Specifically, if $\underline{x}$ now denotes metabolized dose, and $\underline{d}$ denotes administered dose, then $\underline{x}$ and $\underline{d}$ are related by some function $x = h(d)$. Gehring, Watanabe, and Park (1979) have used Michaelis-Menten kinetics to relate x and d. In the absence of data that bear directly on the relationship, the simpler case of first order kinetics has been used here to model the compound uptake by bacterial cells,

$$x = h(d) = A\,[1 - \exp(-Vd)];\ A, V, d \geq 0\ . \qquad (8)$$

Substituting (8) in (7) and absorbing the constant A in S and T yields

$$m(d) = \{B + S[1 - \exp(-Vd)]\} \exp\{-T[1-\exp(-Vd)]\}$$

This may be reparametrized to express the initial slope as a single parameter,

$$m(d) = \{B + V\,[1-\exp(-Sd/V)]\} \exp\{-T[1-\exp(-Sd/V)]\} \qquad (9)$$

Usually the full generality of (9) is neither required nor desirable. Attempts to estimate parameters that are not required to fit the data can lead to high correlation between estimates and can result in inefficient estimation of the parameter (S) of primary interest.

The model (9) with T = 0 corresponds to an underlying dose response curve that initially appears linear, then gradually levels off but does not decrease (Figure 1c).

For data sets extending just up to the shoulder of the curve (the dashed vertical line in Figures 1a, 1b, and 1c), it is not clear on the basis of mutagenicity data alone whether or not the leveling off is due to cell saturation or toxicity. In such a case, independent toxicity experiments can be a useful guide in selecting the more biologically plausible model. Examples of this are given in the next section.

For the relatively rare case in which a higher order (e.g., quadratic) effect is evident at lower doses (e.g., Wei et al., 1980), recourse can be had

to the full parametrization (1) of Stead et al., or to (9) with $\underline{d}$ replaced by a power of $\underline{d}$ or a polynomial in $\underline{d}$.

In summary, a probability model for Salmonella plate assay data can be based on the negative binomial probability distribution for the number of revertants. The utility of this approach is enhanced by the availability of several parametrizations of the mean. Most data sets encountered in practice can be adequately fit by equation (7) or (9). If toxicity is not evident then the toxicity parameter T may be set to zero. Selection of the appropriate model can usually be based on inspection of a plot of the data. These ideas are illustrated in the next section.

C8

SECTION 4
INTERPRETATION OF RESULTS

The feasibility of objectively evaluating Salmonella plate assay data within the framework of a formal statistical model has already been demonstrated by Stead et al. (1980). The present work offers increased flexibility through a more general variance structure and additional biologically plausible parametrizations of the mean number of revertants. Several Ames test data sets will be reviewed here, to illustrate the application of the negative binomial models. Some of the data sets raise questions of model selection that require information beyond that yielded by the standard mutagenicity assay. Independent toxicity data shed some light on these matters.

The Salmonella plate assay data sets, which will be used to illustrate the application of the negative binomial models, are from two studies. The first study tested six fractionated components (acid, base, tar, polar neutrals (PN), nonpolar neutrals (NPN), and polynuclear aromatics (PNA)) of a sample of Illinois coal, using strain TA98. The second study tested six pure vapor phase compounds (dibromoethane, dichloroethane, ethylene oxide, styrene oxide, butylene oxide, and propylene oxide) with strain TA100. The Illinois study employed metabolic activation via S-9 liver homogenate. The pure compound study did not use metabolic activation. Both assays used pour plate procedures. Triplicate plates were used at each of five nonzero doses of the compound, as well as triplicate controls with solvent (DMSO) and triplicate controls without solvent.

ESTIMATION OF C

For both studies, the mean and variance of replicate plates at each dose was computed. Plots of variance versus mean for each study are given in Figures 2a (Illinois) and 2b (pure compounds). Different symbols are plotted for different compounds. In each case it is apparent that the variance tends to exceed the mean. The model of equation (6), variance = $C(\text{mean}^2)$ + mean, was

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ETYPE=M

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: Y

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	1	246386483.11163753	246386483.11163753	16.12	0.0002	0.272620	253.5027
ERROR	43	657384933.22169590	15288033.33073711		STD DEV		Y MEAN
UNCORRECTED TOTAL	44	903771916.33333330			3909.99147651		1542.38636364

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
M2	1	246386483.11163748	16.12	0.0002	1	246386483.11163742	16.12	0.0002

PARAMETER	ESTIMATE	T FOR H0: PARAMETER=0	PR > |T|	STD ERROR OF ESTIMATE
M2	0.00422687	4.01	0.0002	0.00105240

ETYPE=Y

PLOT OF VARIANCE VS MEAN

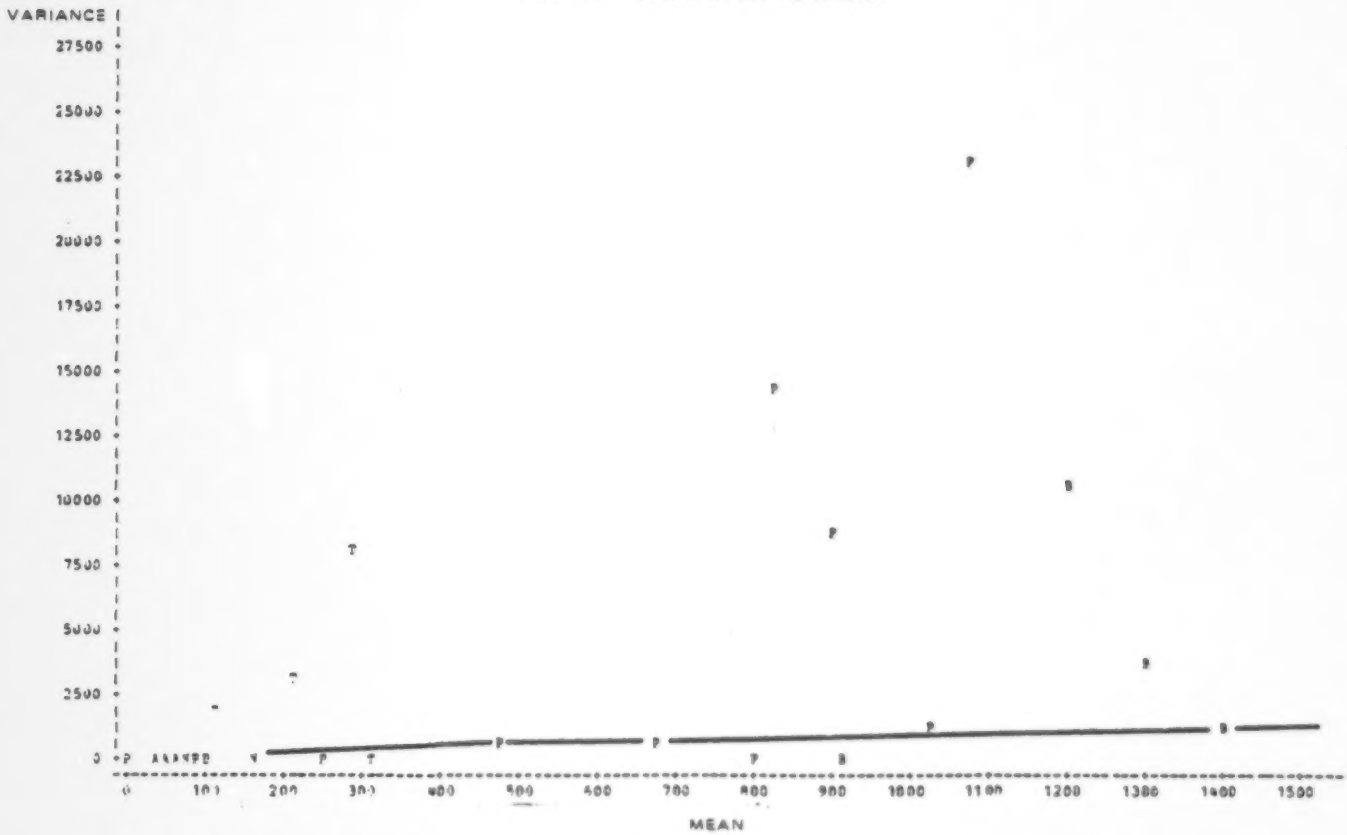

Figure 2a. The variance versus mean relationship for triplicate plates from Illinois coal fractionation Study. Z = variance - mean, M2 = mean^2. The line corresponds to variance equal to mean. The first letter of each fraction is plotted (Acid, Base, Nonpolar Neutral, Polar Neutral, Polynuclear Aromatic, Tar).

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: Z

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	1	26894350.28785609	26894350.28785609	21.01	0.0001	0.350123	205.9839
ERROR	39	49919613.71214392	1279990.09518318		STD DEV		Z MEAN
UNCORRECTED TOTAL	40	76813964.00000001			1131.36647254		549.25000000

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
M2	1	26894350.28785609	21.01	0.0001	1	26894350.28785609	21.01	0.0001

PARAMETER	ESTIMATE	T FOR H0: PARAMETER=0	PR > |T|	STD ERROR OF ESTIMATE
M2	0.01549609	4.58	0.0001	0.00338061

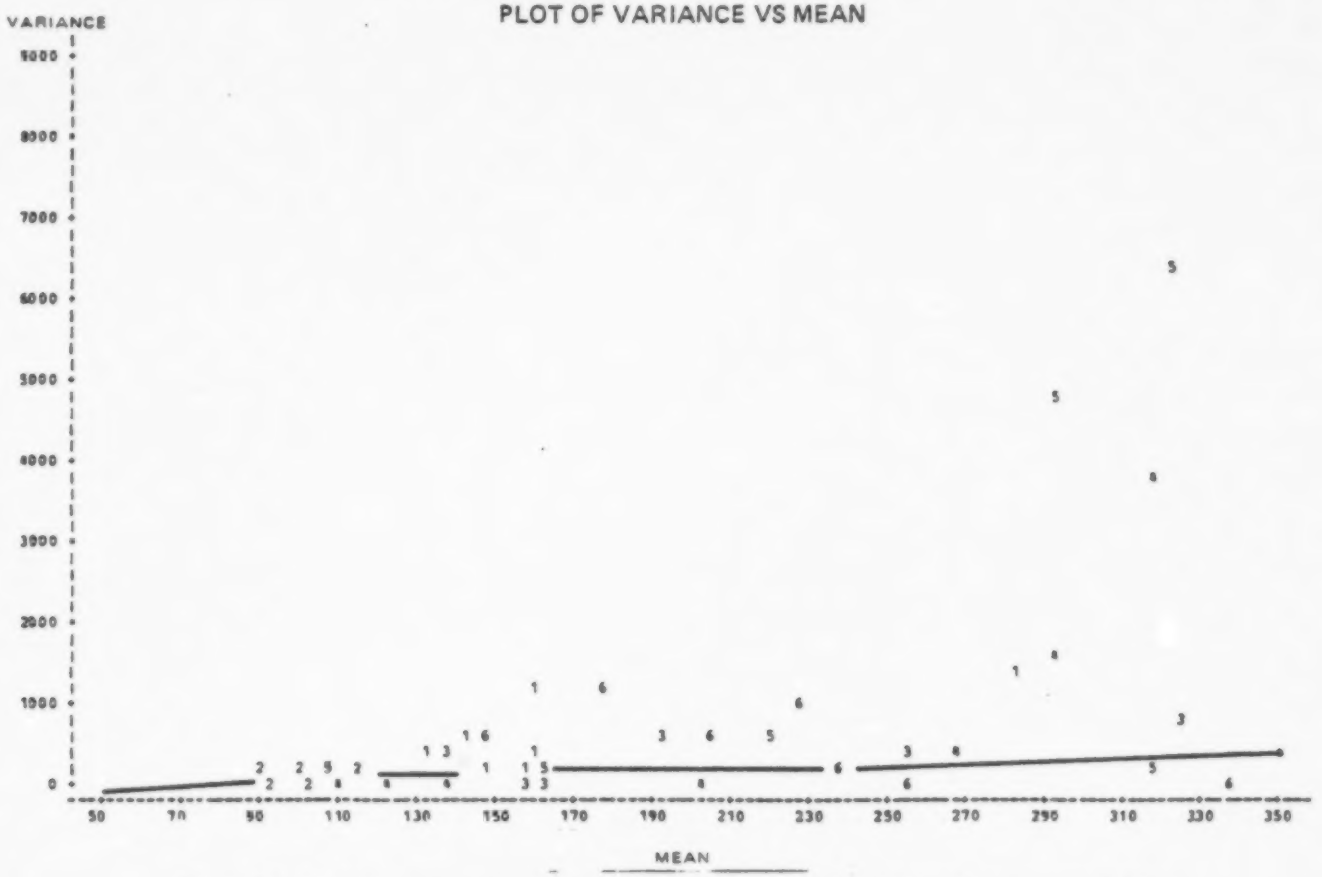

Figure 2b. Variance versus mean relationship for triplicate plates from the pure vapor phase compound study. Z = variance - mean, M2 = mean^2. The line corresponds to variance = mean. Plotted symbols are compound numbers (see text).

fit to each data set by regressing Z = (variance - mean) on M2 = mean^2 using the Statistical Analysis System General Linear Models procedure with zero intercept. The resulting estimates of the parameter C are C = .0042 (standard error .0011) for the Illinois study and C = .0155 (standard error .0034) for the pure compound study. In both cases the hypothesis that C = 0 (i.e., the Poisson case) is rejected at a very significant level ($P < .001$). Because the data indicate that the variance of replicate plates exceeds the mean, we regard the negative binomial models with the above estimates of C as better approximations to reality for the respective Illinois coal fractionation and pure compound data sets. The following analyses of mutagenicity data employ these negative binomial models. The computational procedures are discussed in the Appendix.

ANALYSES OF MUTAGENICITY

Hypothesis tests are at the 5 percent significance level. The first two analyses involve compounds that were judged nonmutagenic. The parametrization (7) was used for the mean in both cases. The parameter estimates for the acid fraction (Figure 3) of the Illinois coal sample are R = 44 for the background number of revertants (standard error SE = 2.9), T = .0005 (SE = .0005) for the toxicity parameter, and S = .057 for the initial slope. R = B in the notation of Section 3. The suggestion of mutagenicity is not judged statistically significant because a 95 percent confidence interval for the initial slope extends from -.047 to .162, which does not exclude S = 0.

The results for the pure compound dichloroethane (C = .0155) are presented in Figure 4. The estimate of the initial slope is exactly zero because the computer program restricts the slope to nonnegative values, and the data exhibit a slightly decreasing trend.

Figures 5a and 5b involve the pure compound butylene oxide and illustrate how the mutagenicity decision can be model dependent. The suggestion of mutagenicity is not statistically significant if the Poisson model (C = 0) is employed, because the confidence interval for the initial slope S(-.001 to .197) includes zero. The parametrization (7) was used for the mean number of revertants. However, using the same parametrization of the mean, with the estimate C = .0155 (Figure 2b) in the negative binomial model, the confidence interval for the initial slope S excludes zero (.001 to .193), so that the

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S T A T I S T I C A L A N A L Y S I S S Y S T E M
COMP=ACID

NON-LINEAR LEAST SQUARES SUMMARY STATISTICS DEPENDENT VARIABLE Y

SOURCE	DF	WEIGHTED SS	WEIGHTED MS
REGRESSION	3	850.23717456	283.41239152
RESIDUAL	18	29.05737719	1.61429873
UNCORRECTED TOTAL	21	879.29455174	
(CORRECTED TOTAL)	20	39.43295614	

PARAMETER	ESTIMATE	ASYMPTOTIC STD. ERROR	ASYMPTOTIC 95 % CONFIDENCE INTERVAL LOWER	UPPER
B	43.63591215	2.93387356	37.47211325	49.79971105
S	0.05742240	1.04988149	-0.04737403	0.16221882
T	0.00054392	0.00053428	-0.00057855	0.00166640

ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS

	B	S	T
B	1.000000	-0.983473	-0.902719
S	-0.983473	1.000000	0.981089
T	-0.902719	0.981089	1.000000

S T A T I S T I C A L A N A L Y S I S S Y S T E M
COMP=ACID

PLOT OF Y*X SYMBOL USED IS O
PLOT OF YHAT*X SYMBOL USED IS P

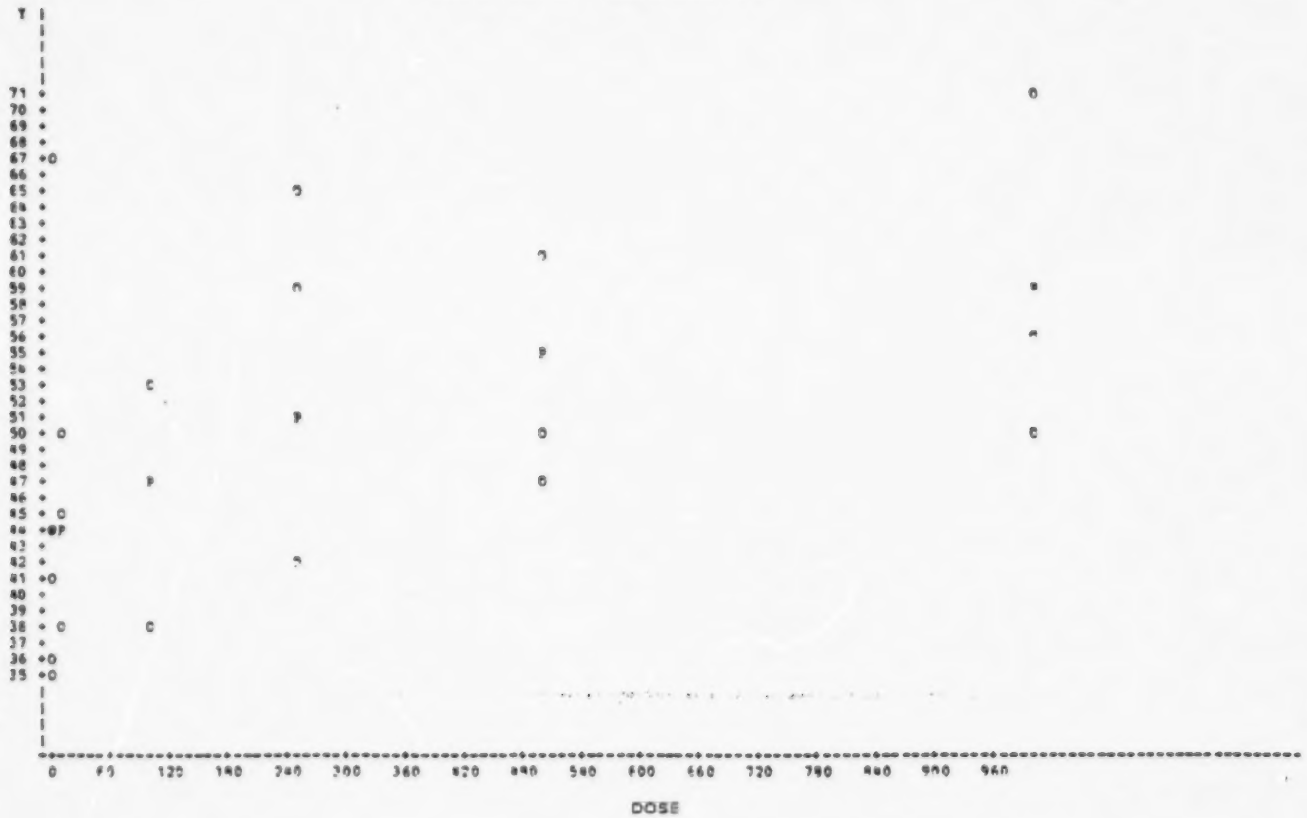

Figure 3. Analysis of the Illinois coal acid fraction.

NON-LINEAR LEAST SQUARES SUMMARY STATISTICS DEPENDENT VARIABLE Y

SOURCE	DF	WEIGHTED SS	WEIGHTED MS
REGRESSION	2	1626.68901469	813.344507350
RESIDUAL	15	15.95503056	1.063668870
UNCORRECTED TOTAL	17	1642.64404525	
(CORRECTED TOTAL)	16	16.22626136	

PARAMETER	ESTIMATE	ASYMPTOTIC STD. ERROR	ASYMPTOTIC 95 % CONFIDENCE INTERVAL: LOWER	UPPER
R	135.9815294	4.812439815	125.7243107	146.2387481
S	0.00000000	0.01001077	-0.02135057	0.02135057
T	0.00003855	0.00000000	0.00003855	0.00003855

ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS

	R	S	T
R	1.000000	-0.691714	0.000000
S	-0.691714	1.000000	0.000000
T	0.000000	0.000000	0.000000

PLOT OF Y*X SYMBOL USED IS O
PLOT OF YHAT*X SYMBOL USED IS P

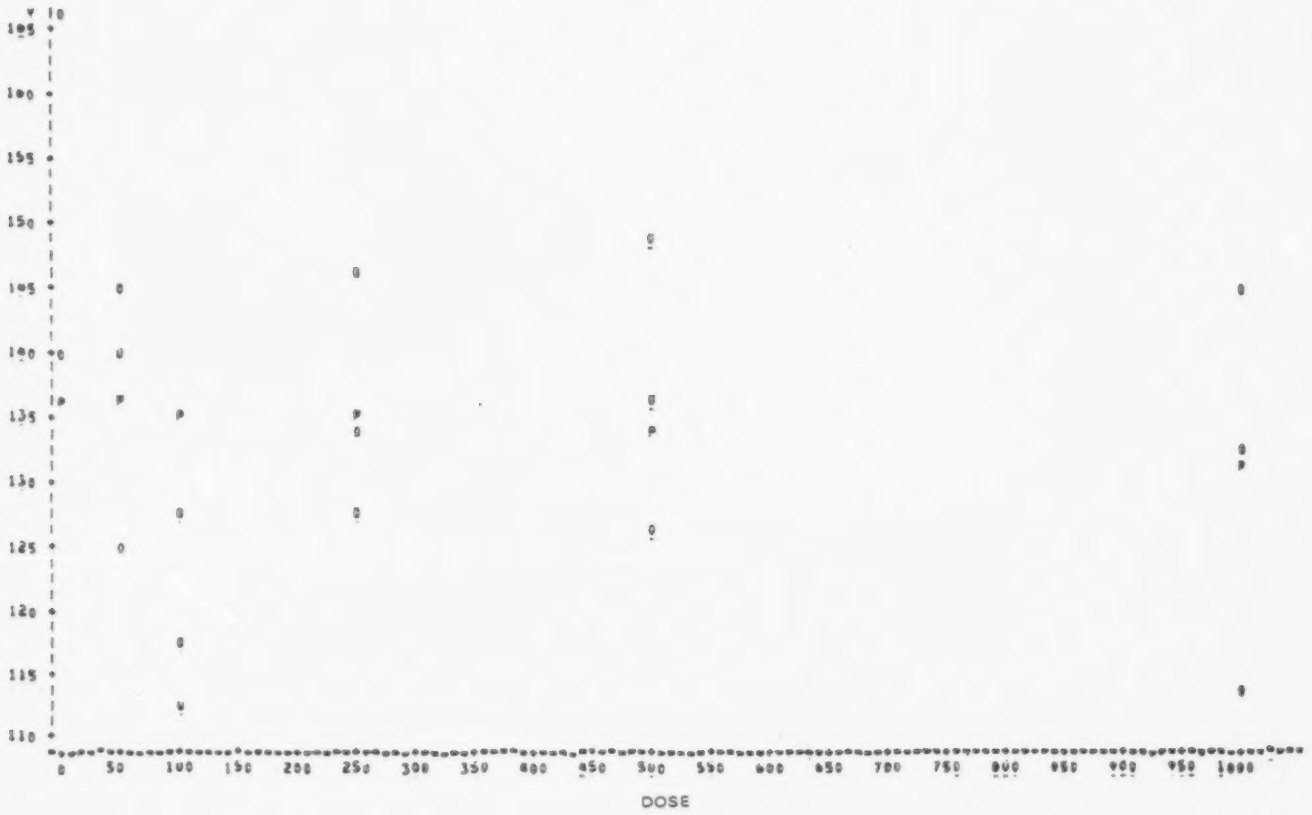

Figure 4. Analysis of the pure compound dichloroethane.

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NON-LINEAR LEAST SQUARES SUMMARY STATISTICS DEPENDENT VARIABLE Y

SOURCE	DF	WEIGHTED SS	WEIGHTED MS
REGRESSION	3	4584.55317171	1528.18439057
RESIDUAL	18	192.68929629	10.70496091
UNCORRECTED TOTAL	21	4777.24246801	
(CORRECTED TOTAL)	20	926.21382831	

PARAMETER	ESTIMATE	ASYMPTOTIC STD. ERROR	ASYMPTOTIC 95 % CONFIDENCE INTERVAL LOWER	UPPER
R	108.94571158	13.45910119	79.82901283	138.06240033
S	0.09818543	0.04728422	-0.00107033	0.19744120
T	0.00006593	0.00013183	-0.00021104	0.00034290

ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS

	R	S	T
R	1.000000	-0.817637	-0.295144
S	-0.817637	1.000000	0.976583
T	-0.295144	0.976583	1.000000

PLOT OF Y*X SYMBOL USED IS O
PLOT OF YHAT*X SYMBOL USED IS P

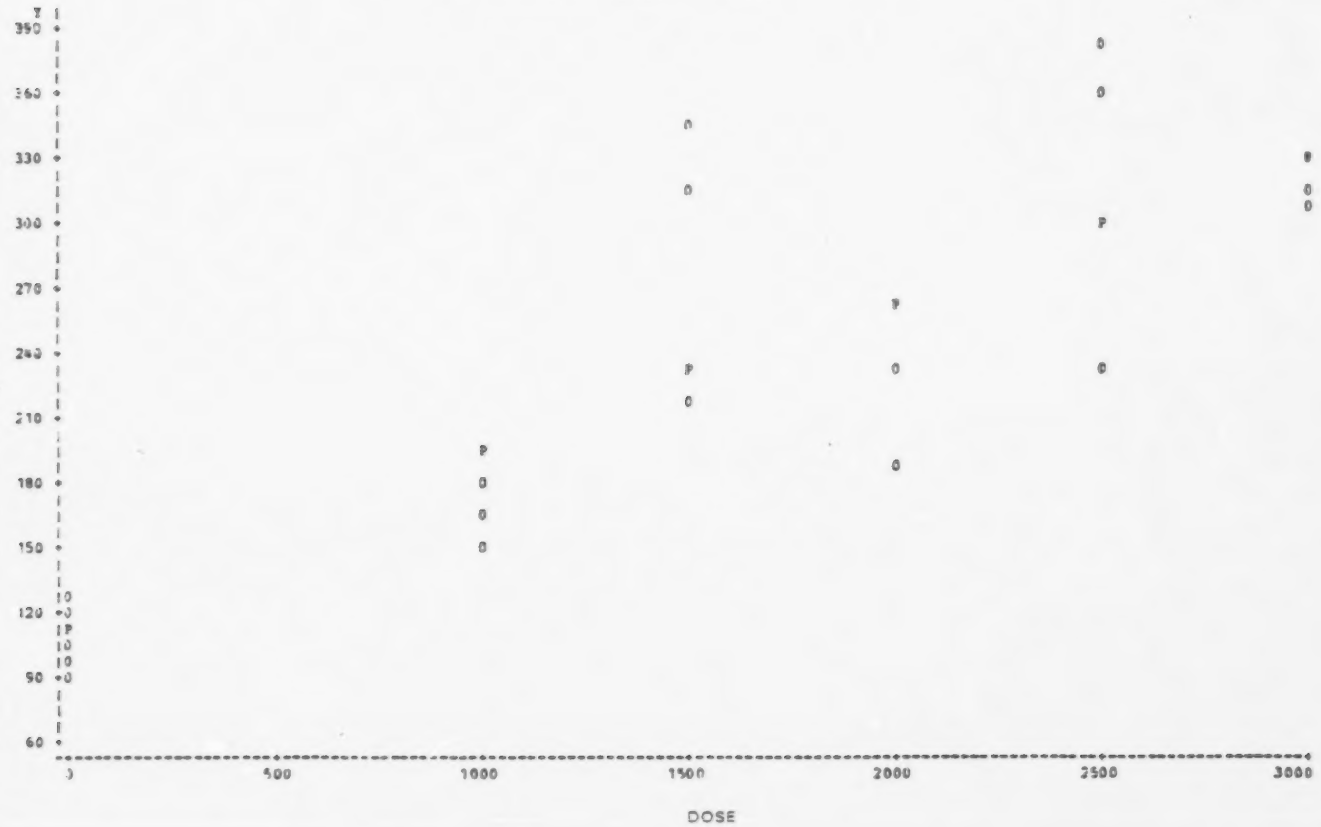

Figure 5a. Analysis of butylene oxide with the Poisson model (C = 0).

CMU=5

NON-LINEAR LEAST SQUARES SUMMARY STATISTICS DEPENDENT VARIABLE Y

SOURCE	DF	WEIGHTED SS	WEIGHTED MS
REGRESSION	3	1016.425965176	338.80866392
RESIDUAL	18	41.10882341	2.28382352
UNCORRECTED TOTAL	21	1057.53481514	
(CORRECTED TOTAL)	20	213.46194866	

PARAMETER	ESTIMATE	ASYMPTOTIC STD. ERROR	ASYMPTOTIC 95 % CONFIDENCE INTERVAL LOWER	UPPER
R	109.25263703	10.54649288	87.09542610	131.40984795
S	0.09718055	0.08582877	-0.00090651	0.19347060
T	0.00006429	0.00013396	-0.00021516	0.00034773

ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS

	R	S	T
R	1.000000	-0.338521	-0.233452
S	-0.338521	1.000000	0.976520
T	-0.233452	0.976520	1.000000

CMU=5

PLOT OF Y*X SYMBOL USED IS 0
PLOT OF YHAT*X SYMBOL USED IS P

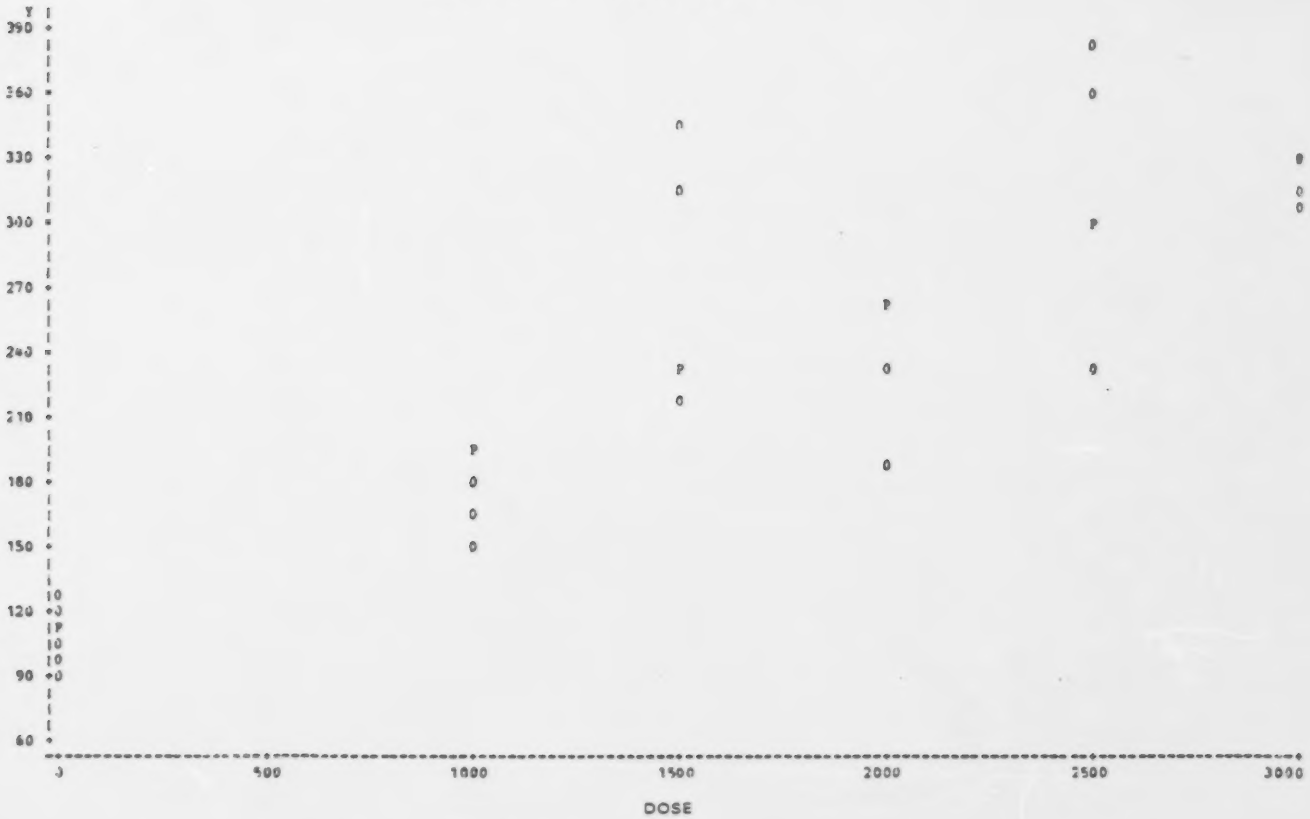

Figure 5b. Analysis of butylene oxide with the negative binomial model (C = .0155).

compound is judged mutagenic. The negative binomial model anticipates and accounts for more variance than the Poisson model. In general, however, the Poisson and negative binomial models seem to give very similar estimates of parameters and standard errors. Of course, in both of these instances the decisions are very marginal and further experimentation is warranted. This compound was also tested using a preincubation procedure on log phase cells and was more obviously mutagenic than on the basis of the data reviewed here. Those results have been presented elsewhere (Wolff et al., 1980).

The next example illustrates the possible influence of outliers and their disposition. Statistical analysis of the tar fraction from the Illinois coal sample, using parametrization (7), is presented in Figure 6a. The three data points at a dose of 10 μg appear discrepant, averaging about 300 revertants while the value predicted by the model is about 130 revertants. The confidence interval for the initial slope, using parametrization (7) in the negative binomial model with C = .0042, nearly includes zero (.19 to 5.19). The analyses were rerun (Figure 6b) without the data points at the dose of 10, which produced more than a 50 percent decrease in all three standard errors and made the test of mutagenicity appear much more significant. In this particular case the model and estimation procedure were robust to the outlier, i.e. very similar parameter estimates were obtained with or without the outliers. Outlier rejection is, at best, a shaky business. Robust fitting procedures have been particularly useful in other applications and may serve well for Salmonella plate assay data.

Results from the base fraction of the Illinois coal are in Figures 7a and 7b. Regarding selection of a parametrization for the mean m(x), any of the following possibilities could be suggested by the plot of observed values (0's in Figure 7a).

1. The three points at a dose of 250 are slightly in error and overestimate the true value. Toxicity is not in evidence. The curve levels off, due to saturation of the bacterial cells with the compound. The model (9) with T = 0 could be used (Figure 7a).
2. The three points at a dose of 500 are underestimates, and the parametrization (7) with T > 0 is appropriate.

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NON-LINEAR LEAST SQUARES SUMMARY STATISTICS DEPENDENT VARIABLE Y

SOURCE	DF	WEIGHTED SS	WEIGHTED MS
REGRESSION	3	1837.78472621	612.59491207
RESIDUAL	18	755.91543741	41.99530208
UNCORRECTED TOTAL	21	2593.70017362	
(CORRECTED TOTAL)	20	1291.25163352	

PARAMETER	ESTIMATE	ASYMPTOTIC STD. ERROR	ASYMPTOTIC 95 % CONFIDENCE INTERVAL LOWER	UPPER
R	109.76266492	29.28809791	48.23106109	171.29426875
S	2.69194335	1.19024238	0.19137341	5.19255329
T	0.00440235	0.00088437	0.00254438	0.00626033

ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS

	R	S	T
R	1.000000	-0.292658	-0.188626
S	-0.292658	1.000000	0.843918
T	-0.188626	0.843918	1.000000

PLOT OF Y*I SYMBOL USED IS C
PLOT OF YHAT*I SYMBOL USED IS P

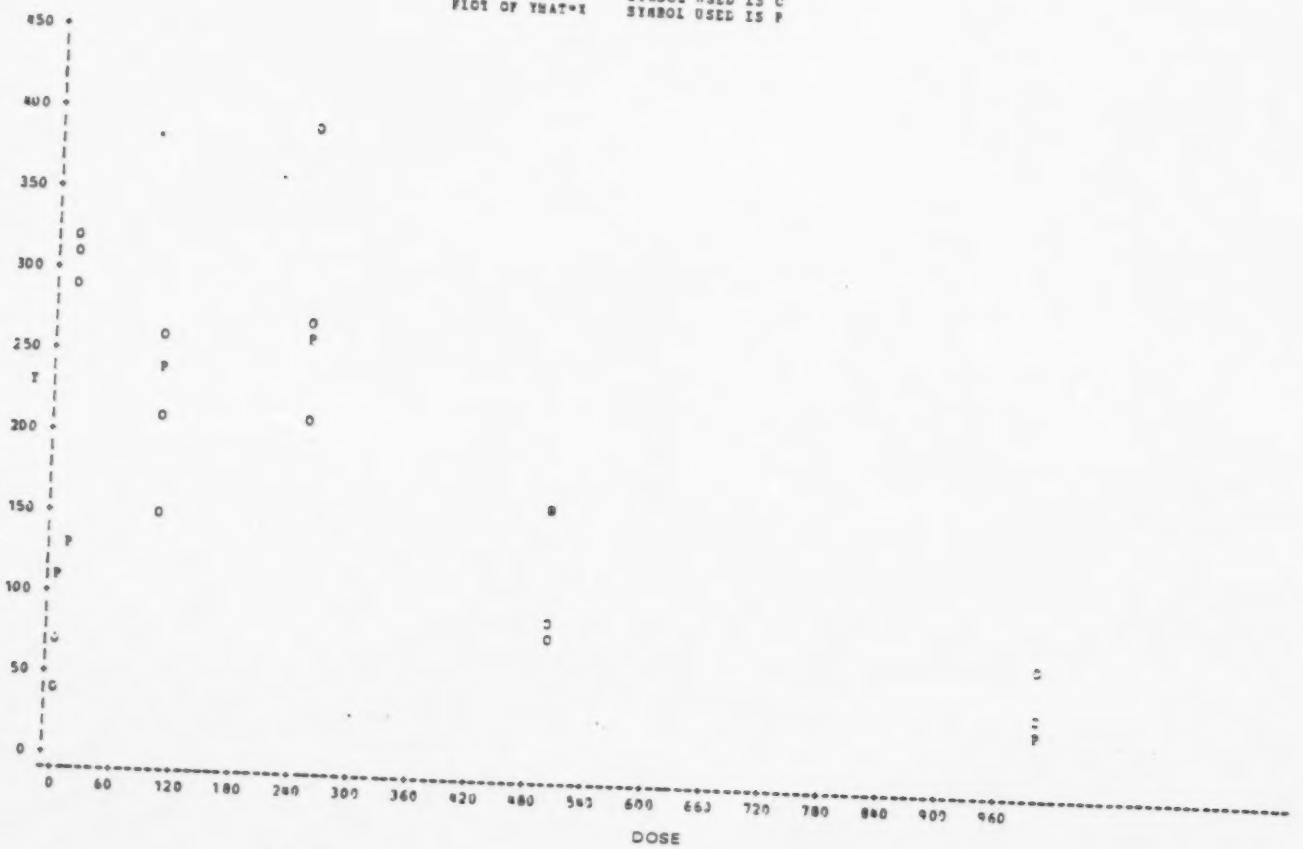

Figure 6a. Analysis of the Illinois coal tar fraction.

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NON-LINEAR LEAST SQUARES SUMMARY STATISTICS DEPENDENT VARIABLE Y

SOURCE	DF	WEIGHTED SS	WEIGHTED MS
REGRESSION	3	1308.02281123	436.00760374
RESIDUAL	15	127.17914886	8.47860992
UNCORRECTED TOTAL	18	1444.20195610	
(CORRECTED TOTAL)	17	635.44097985	

PARAMETER	ESTIMATE	ASYMPTOTIC STD. ERROR	ASYMPTOTIC 95 % CONFIDENCE INTERVAL LOWER	UPPER
R	44.28256021	8.89881237	25.33068108	63.23443934
S	2.73467599	0.47681651	1.71922210	3.75012989
T	0.00430925	0.00037722	0.00350528	0.00511326

ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS

	R	S	T
R	1.000000	-0.157264	-0.085750
S	-0.157264	1.000000	0.819806
T	-0.085750	0.819806	1.000000

PLOT OF Y*X SYMBOL USED IS O
PLOT OF YHAT*X SYMBOL USED IS P

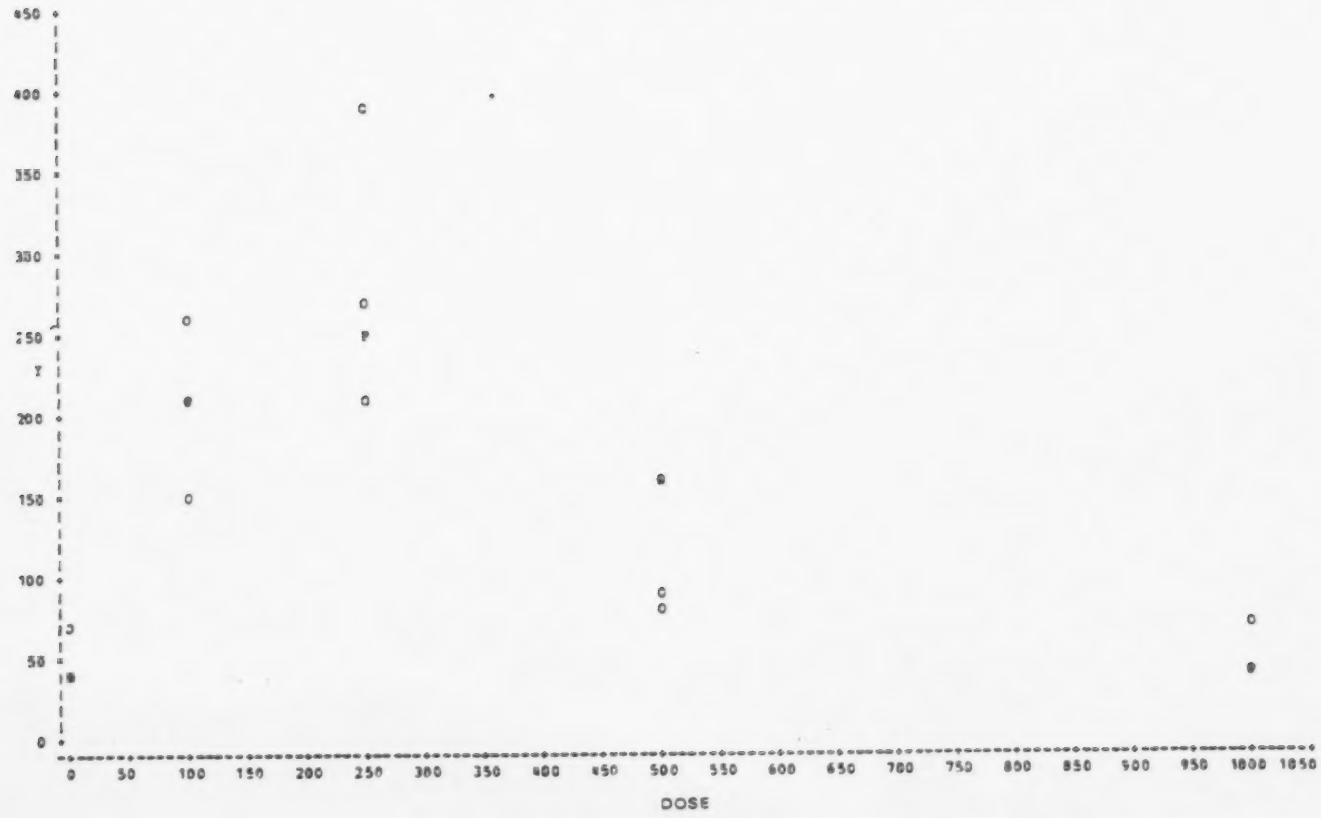

Figure 6b. Analysis of the Illinois coal tar fraction, with dose = 10 deleted.

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NON-LINEAR LEAST SQUARES SUMMARY STATISTICS DEPENDENT VARIABLE Y

SOURCE	DF	WEIGHTED SS	WEIGHTED MS
REGRESSION	3	2840.82115671	946.80705224
RESIDUAL	18	93.28733242	5.18262958
UNCORRECTED TOTAL	21	2933.70848913	
(CORRECTED TOTAL)	20	2362.61870786	

PARAMETER	ESTIMATE	ASYMPTOTIC STD. ERROR	ASYMPTOTIC 95 % CONFIDENCE INTERVAL LOWER	UPPER
A	39.55081318	6.18936231	26.54757339	52.55405269
S	11.32884350	1.45811780	8.27347555	14.38421145
B	1329.95976240	86.07305259	1149.05818400	1510.72134080

ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS

	A	S	B
A	1.000000	-0.231788	-0.012077
S	-0.231788	1.000000	-0.351787
B	-0.012077	-0.351787	1.000000

PLOT OF Y*X SYMBOL USED IS O
PLOT OF YHAT*X SYMBOL USED IS P

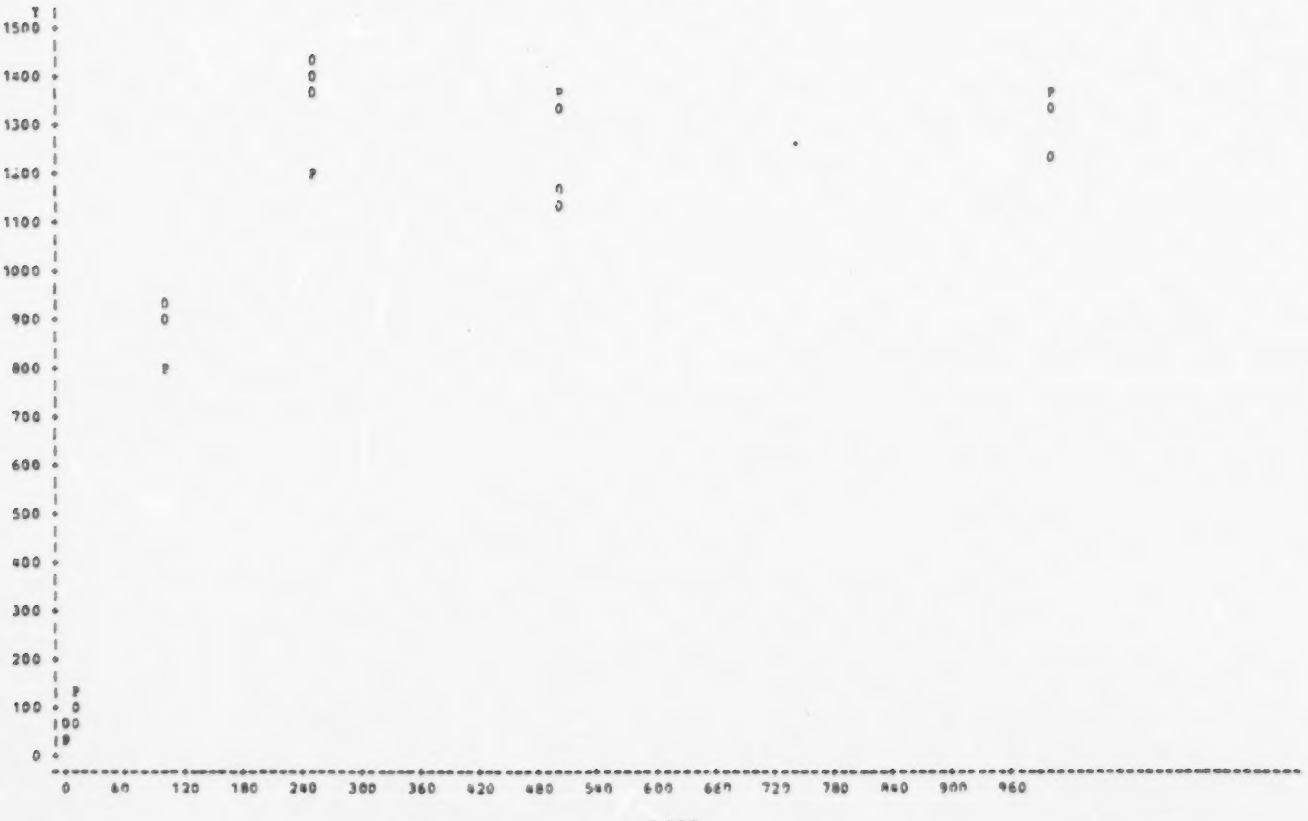

Figure 7a. Analysis of the Illinois coal base fraction using equation (9) with T = 0.

COMP=BASE ETYPE=I

PLOT OF P1*DOSE LEGEND: A = 1 OBS, B = 2 OBS, ETC.
PLOT OF P2*DOSE LEGEND: A = 1 OBS, B = 2 OBS, ETC.
PLOT OF P3*DOSE LEGEND: A = 1 OBS, B = 2 OBS, ETC.

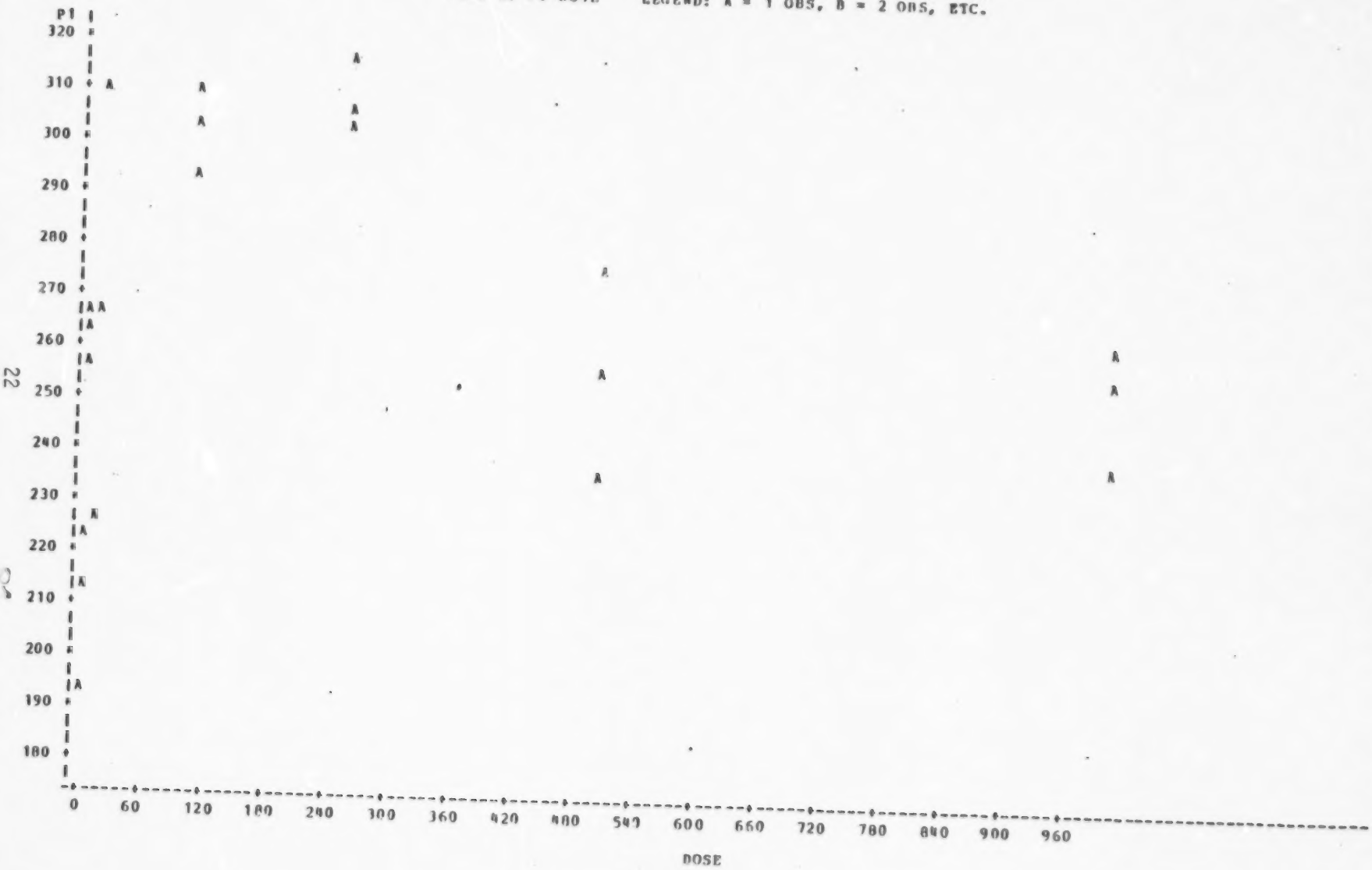

Figure 7b. Toxicity data for the Illinois coal base fraction.

3. The triples of points at each dose provide unbiased estimates of the true mean number of revertants. Toxicity operates to produce the drop between doses of 250 and 500. Saturation of the bacterial cells occurs near the dose of 500, and the curve levels off. The parametrization (9) with T = 0 was developed for this situation.

The data from an independent, concurrent toxicity experiment are shown in Figure 7b. The experiment is done under conditions identical to the regular Salmonella plate assay, except that the target number of plated cells is approximately 250, rather than $5 \cdot 10^8$, and ample histidene is provided for repeated cell division. The toxicity controls yielding counts of 212, 255, 264 were with solvent (DMSO); the other 3 controls, 194, 265, 224 were with cells only (no solvent). The respective means are 244 (dose = 0; with DMSO), 267 (dose = 10), 302 (dose = 100), 309 (dose = 250), 256 (dose = 500), 254 (dose = 1,000). Overall, we interpret the compound as nontoxic and select the first option above, i.e., the saturation model (9) without toxicity. The corresponding statistics are given in Figure 7a.

The final example involves the polar neutral (PN) fraction of the Illinois coal sample. The analyses using the parametrization (7) are presented in Figure 8a. This model, incorporating toxicity through the factor exp (-Tx), assumes that toxicity is operating over the entire dose range, and that its effect is increasing differentiably. The accompanying toxicity data for this experiment are plotted in Figure 8b, which strongly suggests a toxicity threshold. Toxicity is not evident until somewhere above the dose of 50. Thus it appears that neither of the available models properly accounts for toxicity. Work is in progress on this problem. A temporary solution is to analyze the data only in the dose range exhibiting no toxicity, using the kinetic model (9) without toxicity (T = 0). The results of this analysis are presented in Figure 8c. Analyzed either way, the polar neutral fraction is clearly mutagenic.

NON-LINEAR LEAST SQUARES SUMMARY STATISTICS DEPENDENT VARIABLE Y

SOURCE	DF	WEIGHTED SS	WEIGHTED MS
REGRESSION	3	10501.25163403	3500.41721134
RESIDUAL	18	546.05562132	30.33642341
UNCORRECTED TOTAL	21	11047.30725536	
(CORRECTED TOTAL)	20	9337.87890588	

PARAMETER	ESTIMATE	ASYMPTOTIC STD. ERROR	ASYMPTOTIC 95 % CONFIDENCE INTERVAL LOWER	UPPER
R	45.21363775	15.06070915	13.57247102	76.85480448
S	67.32175402	6.13544055	54.43175692	80.21175113
T	0.02279324	0.00138011	0.01989376	0.02569272

ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS

	R	S	T
R	1.000000	-0.149804	-0.079725
S	-0.149804	1.000000	0.784680
T	-0.079725	0.784680	1.000000

PLOT OF Y*I SYMBOL USED IS O
PLOT OF YHAT*I SYMBOL USED IS P

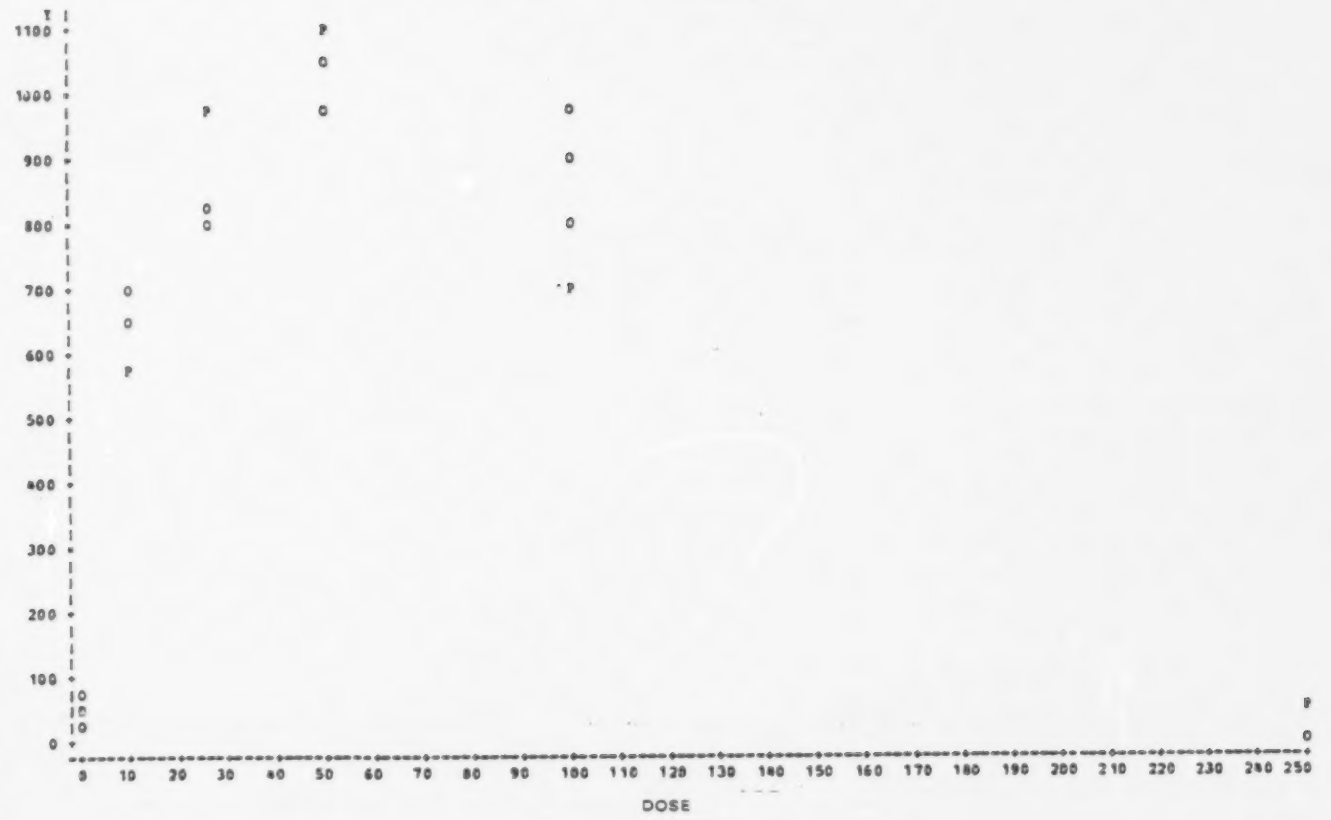

Figure 8a. Analysis of the Illinois coal polar neutral fraction, using equation (7).

COMP=PN ETYPE=T

PLOT OF P1*DOSE LEGEND: A = 1 OBS, B = 2 OBS, ETC.
PLOT OF P2*DOSE LEGEND: A = 1 OBS, B = 2 OBS, ETC.
PLOT OF P3*DOSE LEGEND: A = 1 OBS, B = 2 OBS, ETC.

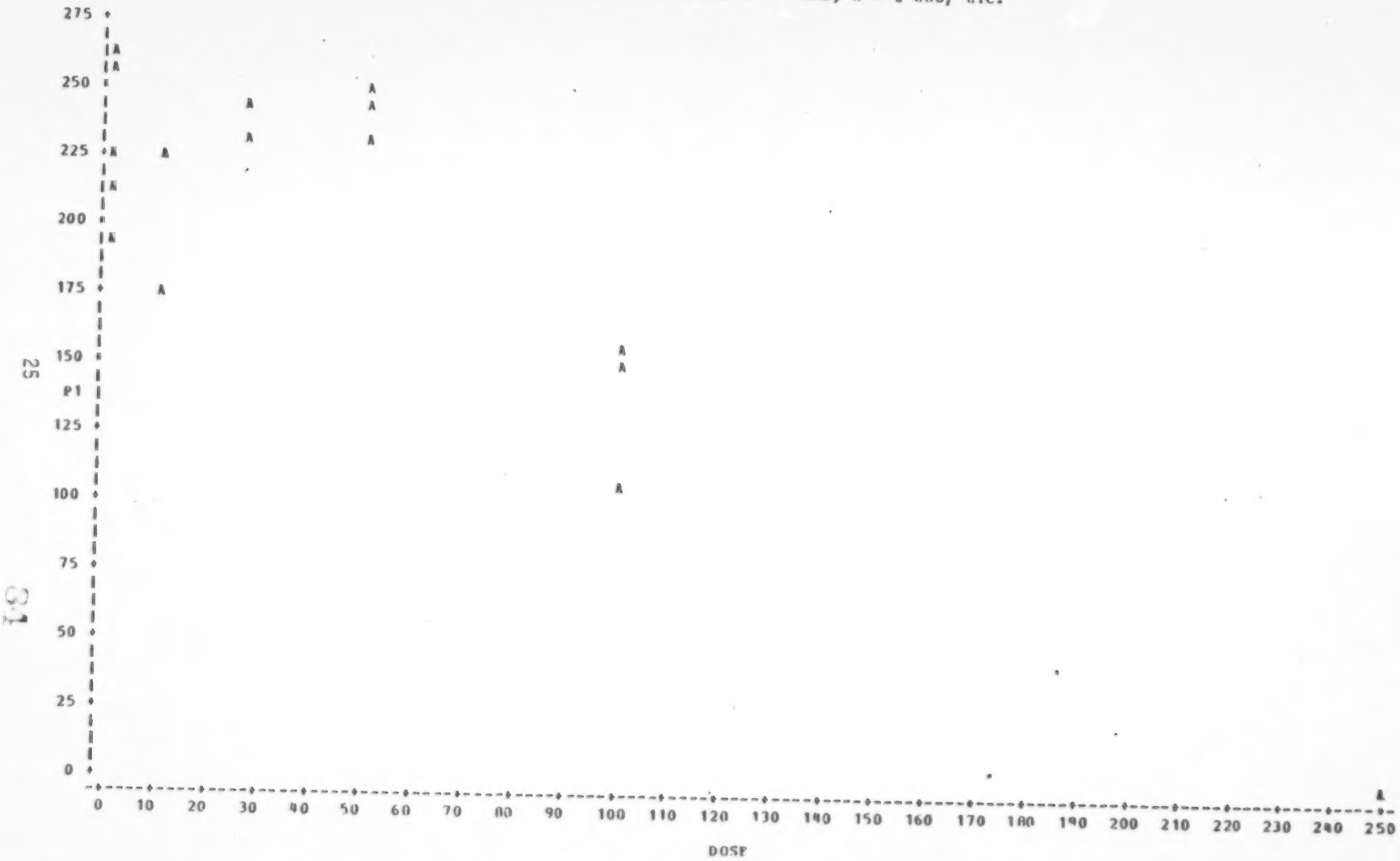

Figure 8b. Toxicity data for the Illinois coal polar neutral fraction.

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NON-LINEAR LEAST SQUARES SUMMARY STATISTICS DEPENDENT VARIABLE Y

SOURCE	DF	WEIGHTED SS	WEIGHTED MS
REGRESSION	3	1882.98250574	627.66750325
RESIDUAL	12	24.22370667	2.01864222
UNCORRECTED TOTAL	15	1907.16621682	
(CORRECTED TOTAL)	14	1879.12614852	

PARAMETER	ESTIMATE	ASYMPTOTIC STD. ERROR	ASYMPTOTIC 95 % CONFIDENCE INTERVAL LOWER	UPPER
R	44.05811958	4.19087177	34.92298095	53.18525821
S	98.28422606	12.98775623	69.94629448	126.54215764
B	921.41678766	51.36737080	809.08097306	1033.77260225

ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS

	R	S	B
R	1.000000	-0.066961	-0.076135
S	-0.066961	1.000000	-0.528563
B	-0.076135	-0.528563	1.000000

PLOT OF Y*X SYMBOL USED IS 0
PLOT OF YHAT*X SYMBOL USED IS P

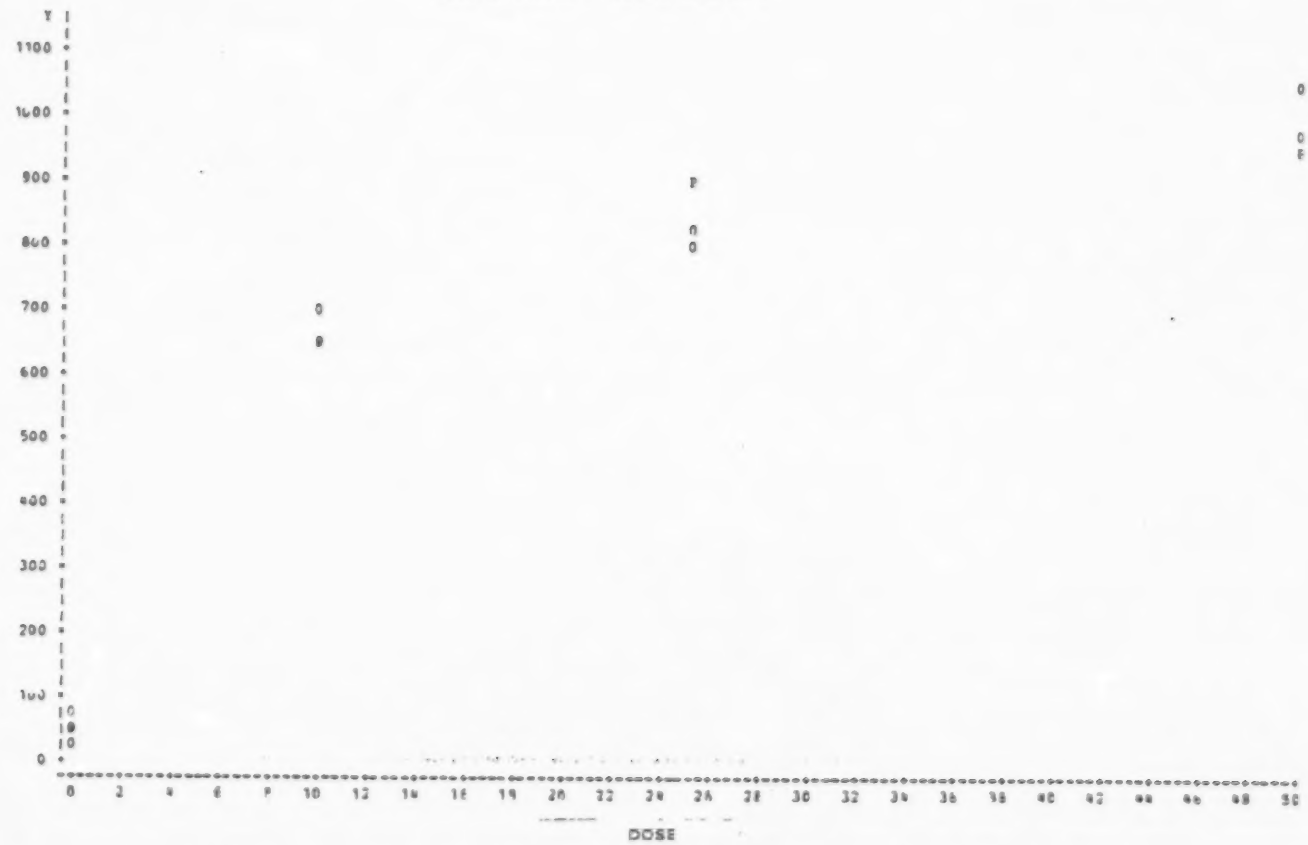

Figure 8c. Analysis of the Illinois coal polar neutral fraction, using the kinetic model (9) without toxicity (T = 0).

SECTION 5
SUMMARY AND DISCUSSION

A biochemically plausible model has been presented for statistical analysis of Salmonella plate assay mutagenicity data. The model employs a negative binomial distribution for the number of revertant bacteria. The negative binomial distribution is obtained by using a Poisson approximation to the binomial distribution for the number of revertants, conditional on the number N of bacteria surviving the initial plating. The N survivors are regarded as the population available to reverse mutate or die of toxic effects of the tested compound. Because of the unavoidable plate-to-plate variation in N, it is reasonable to treat N as a random quantity. The family of gamma distributions was chosen to represent the probability distribution of N. Averaging the Poisson distribution over the gamma distributed N results in a negative binomial distribution for the unconditional number of revertants.

The parameters of the negative binomial distribution include the two parameters (shape a and scale b) of the gamma distribution, as well as the probability p(x) at dose x of surviving toxic effects and reverting back to the wild type. The scale parameter b can be eliminated by reparametrizing in terms of the mean $m(x) = abp(x)$ as in equations (7) and (8). Parametrizations of varying complexity have been developed for the mean m(x). The most useful parametrization (equation 7) is a linear function of dose attenuated by an exponentially decreasing term to account for toxicity.

The approach developed here for statistical analysis of Salmonella plate assay data offers several advantages. The negative binomial model is biologically based and its parameters have clear interpretations: B is the mean number of spontaneous revertants; S is the initial rate of increase in the mean number of revertants as a function of dose; and T incorporates toxicity. With several parametrizations of the mean available, the model is sufficiently flexible to accommodate the variety of Ames test data sets encountered in

practice, and accounts for the well-documented phenomenon of variance-greater-than-mean among replicates at mean levels above 100 revertants per plate.

The most important feature of the methods presented here and in Stead et al. (1980) is increased objectivity in data interpretation. Salmonella plate assay data have been interpreted according to rather arbitrary rules in the past. In the present scheme, once the significance level of the test (e.g., 5 percent) has been chosen, the mutagenicity of the compound is addressed by testing the null hypothesis that the slope S is zero against the alternative hypothesis that S is positive. The compound is regarded as mutagenic if and only if S is significantly greater than zero.

Of course, it is not possible to completely remove subjectivity from the analysis of bioassay data. Because more than one parametrization of the mean is required to fit the wide range of data sets arising in practice, some subjectivity is involved in choosing the parametrization. It may be possible to unify almost all cases in a single parametrization, but the number of parameters required would probably result in frequent convergence problems.

For some Ames test data sets, the underlying dose response shape is clearly perceived, but is ambiguous in that the shape could be obtained from two or more fundamentally different biologic mechanisms. Consider, for example, the three forms in Figure 1, up to the shoulders on the curves (the dashed vertical lines). If the actual doses employed do not extend far enough past this shoulder, then it is not obvious whether a toxicity model or a saturation model is appropriate. In such a case, independent toxicity data can clearly add insight. This will be elaborated in a future manuscript.

APPENDIX
COMPUTATIONS

For parameter estimation and hypothesis testing, the method of maximum likelihood enjoys numerous optimality properties. Maximum likelihood was employed via a Gauss-Newton procedure by Stead et al. (1980) in fitting the Poisson model with mean m(x) as in (1). Maximum likelihood estimation is employed in the present work.

For a broad class of probability distributions (the regular exponential family), it is possible to obtain maximum likelihood estimates via iteratively reweighted nonlinear least squares (IRNLS) regression, by fitting the mean m(x) of equation (8) to the revertant counts, using weights equal to the inverse 1/v(x) of the variance (equation 6) of the number of revertants. Jennrich and Moore (1975) present the necessary theory and several similar applications. The advantage of the approach stems from the wide availability of nonlinear least squares regression packages (such as SAS NLIN, and BMD P3R), which require only a few user-supplied control statements for execution and employ effective search techniques. Output typically includes plots of observed vs. predicted values, parameter estimates, standard errors, and confidence intervals.

A copy of the SAS program used to fit the model (5) with m(x) parametrized as in (7) is given below. The NLIN procedure is so robust with respect to start values that a single admissible set can serve adequately for most data sets. The values B = 50, S = 1, T = .002 have worked in all cases we have tried with strain TA98. For strain 100 the values B = 150, S = 1, and T = .002 suffice.

The quantity $\underline{c} = 1/a$ is treated as a constant in our approach. The problems and inconvenience attendant in regarding it as a parameter are considerable. First, the negative binomial family is no longer a regular exponential family if $\underline{a}$ is treated as a parameter. This invalidates the use of nonlinear iteratively reweighted least squares for obtaining maximum likelihood estimates,

and forces the use of direct maximum likelihood. Some of the related derivatives involve the derivative of the gamma function and require numeric integration within each step of the iteration on parameters. It is thus a considerable simplification to have a reasonable estimate of the parameter a from an independent source, which does not require estimation of a as part of the same iterative scheme that estimates the parameters R, S, and T.

For these reasons we have estimated the parameter c directly from the relationship between variance and mean of replicate plates.

S T A T I S T I C A L A N A L Y S I S S Y S T E M 15:07 SUNDAY, MAY 11, 1980

NOTE: THE JOB HAS BEEN RUN UNDER RELEASE 79.3A OF SAS AT TRIANGLE UNIVERSITIES COMPUTATION CENTER.

```
1          DATA;
2          INFILE IN;
3          INPUT MT $ 1 MIX 4 CNUM 7  PREP $ 8 DOSE 10-15                    00000060
4              P1 17-21 P2 23-27 P3 29-33;                                   00000070
5            IF PREP¬='C' THEN DELETE;                                       00000080
6            IF MT¬='M' THEN DELETE;                                         00000090
7          IF MIX=4 THEN DELETE;                                             00000100
8          IF CNUM¬=5 THEN DELETE;                                           00000110
9          DROP P1-P3;                                                       00000120
10         X=DOSE;Y=P1;OUTPUT;                                               00000130
11         X=DOSE;Y=P2;OUTPUT;                                               00000140
12         X=DOSE;Y=P3;OUTPUT;                                               00000150
                                                                             00000160
                                                                             00000170
```

NOTE: INFILE IN IS:
DSNAME=RTI.C43.PO10497.MYERS.PURE.DATA,
UNIT=DISK,VOL=SER=RTI111,DISP=SHR,
DCB=(BLKSIZE=4240,LRECL=80,RECFM=FB)

NOTE: 250 LINES WERE READ FROM INFILE IN.
NOTE: DATA SET WORK.DATA1 HAS 21 OBSERVATIONS AND 7 VARIABLES. 203 OBS/TRK.
NOTE: THE DATA STATEMENT USED 0.30 SECONDS AND 130K.

```
13         PROC SORT;BY  CNUM;                                               00000180
```

NOTE: DATA SET WORK.DATA1 HAS 21 OBSERVATIONS AND 7 VARIABLES. 203 OBS/TRK.
NOTE: THE PROCEDURE SORT USED 0.99 SECONDS AND 104K.

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14         PROC NLIN ITER=30 METHOD=MARQUARDT;
15         BY CNUM;                                                          00000190
16           PARMS                                                           00000200
17             R=150 S=1 T=.002                                              00000210
18           :BOUNDS R>0 S>0 T>0;                                            00000220
19         C=.0155;                                                          00000230
20           E=EXP(-T*I);U=R*S*X;MEAN=U*E;                                   00000240
21           VAR=MEAN*(C*MEAN+1);                                            00000250
22          MODEL Y=MEAN;                                                    00000260
23           DER.R=E;DER.S=X*E;DER.T=-MEAN*X;                                00000270
24           _WEIGHT_=1/VAR;                                                 00000280
25           OUTPUT PREDICTED=THAT   PARMS= R S T;                           00000290
                                                                             00000300
```

NOTE: A DERIVATIVE FREE METHOD IS AVAILABLE IN THIS RELEASE.
TO TRY IT, JUST OMIT THE DER. STATEMENTS.
NOTE: ABOVE MESSAGE FOR BY GROUP:
CNUM=5
NOTE: DATA SET WORK.DATA2 HAS 21 OBSERVATIONS AND 11 VARIABLES. 167 OBS/TRK.
NOTE: THE PROCEDURE NLIN USED 0.92 SECONDS AND 176K AND PRINTED PAGES 1 TO 2.

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26         PROC SORT; BY CNUM;
27         *PROC PRINT;                                                      00000310
                                                                             00000320
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NOTE: DATA SET WORK.DATA2 HAS 21 OBSERVATIONS AND 11 VARIABLES. 167 OBS/TRK.

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